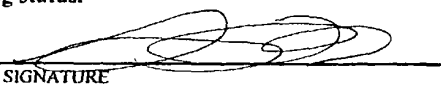


518 Rec'd PCT/PTO 26 JUL 2001

FORM PTO-1390 (REV. 11-2000)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER 266/034	
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371				U.S. APPLICATION NO. (If known, see 37 CFR 1.5 Not Yet Assigned <b>09/890371</b> )	
INTERNATIONAL APPLICATION NO. PCT/EP00/00598		INTERNATIONAL FILING DATE 26 January 2000		PRIORITY DATE CLAIMED 27 January 1999	
TITLE OF INVENTION Transnasal Transport/immunisation with highly adaptable carriers					
APPLICANT(S) FOR DO/EO/US Gregor Ceyc et al					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:					
<p>1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>3. <input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.</p> <p>4. <input checked="" type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (Article 31).</p> <p>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))</p> <p>a. <input checked="" type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau).</p> <p>b. <input type="checkbox"/> has been communicated by the International Bureau.</p> <p>c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</p> <p>6. <input type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).</p> <p>a. <input type="checkbox"/> is attached hereto.</p> <p>b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4).</p> <p>7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))</p> <p>a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau).</p> <p>b. <input type="checkbox"/> have been communicated by the International Bureau.</p> <p>c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</p> <p>d. <input checked="" type="checkbox"/> have not been made and will not be made.</p> <p>8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).</p> <p>9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</p> <p>10. <input type="checkbox"/> An English lanugage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</p>					
Items 11 to 20 below concern document(s) or information included:					
<p>11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</p> <p>12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</p> <p>13. <input type="checkbox"/> A FIRST preliminary amendment.</p> <p>14. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.</p> <p>15. <input type="checkbox"/> A substitute specification.</p> <p>16. <input type="checkbox"/> A change of power of attorney and/or address letter.</p> <p>17. <input type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.</p> <p>18. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4).</p> <p>19. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).</p> <p>20. <input checked="" type="checkbox"/> Other items or information: PCT Cover Page of Published Int'l Application PCT Notification of Transmittal of IPER PCT Notification of Int'l Search Report PCT Request PCT Written Opinion</p>					
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JC18 Rec'd PCT/PTO 26 JUL 2001

U.S. APPLICATION NO. (37 CFR 2.101) <b>09/890371</b>		INTERNATIONAL APPLICATION NO. PCT/EP00/00598		ATTORNEY'S DOCKET NUMBER 266/034		
21. <input type="checkbox"/> The following fees are submitted: <b>BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):</b> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... \$1000.00  International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... \$860.00  International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... \$710.00  International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... \$690.00  International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) ..... \$100.00 <b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>				<b>CALCULATIONS PTO USE ONLY</b>		
				\$ 860.00		
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$ 130.00		
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	\$		
Total claims	- 20 =		x \$18.00	\$		
Independent claims	- 3 =		x \$80.00	\$		
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$270.00	\$		
<b>TOTAL OF ABOVE CALCULATIONS =</b>				\$ 990.00		
<input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				+		
<b>SUBTOTAL =</b>				\$ 990.00		
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$		
<b>TOTAL NATIONAL FEE =</b>				\$		
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +				\$		
<b>TOTAL FEES ENCLOSED =</b>				\$		
				Amount to be refunded:	\$	
				charged:	\$	
a. <input type="checkbox"/> A check in the amount of \$ _____ to cover the above fees is enclosed. b. <input checked="" type="checkbox"/> Please charge my Deposit Account No. <u>12-2475</u> in the amount of \$ <u>990.00</u> to cover the above fees. A duplicate copy of this sheet is enclosed. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. _____. A duplicate copy of this sheet is enclosed. d. <input type="checkbox"/> Fees are to be charged to a credit card. <b>WARNING:</b> Information on this form may become public. <b>Credit card                  information should not be included on this form.</b> Provide credit card information and authorization on PTO-2038.						
NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.						
SEND ALL CORRESPONDENCE TO: Suzanne L. Biggs Lyon & Lyon 633 West Fifth Street, Suite 4700 Los Angeles, CA 90071						
				 SIGNATURE		
				Suzanne L. Biggs NAME		
				30, 158 REGISTRATION NUMBER		

24/PRTS

IDEA AG  
C 2481 PCT

Transnasal transport/immunisation with highly adaptable carriers

The invention deals with the transport of preferably large molecules across nasal mucosa by means of specially designed, highly adaptable carriers loaded with said molecules. One of the purposes of making such formulations is to achieve non-invasive systemic delivery of therapeutic polypeptides, proteins and other macromolecules; the other intent is to overcome circumstantially the blood-brain barrier by exploiting the nasal cavity to enter the body and then to get access to the brain. A third intent is to achieve successful protective or tolerogenic immunisation via nasal antigen or allergen administration.

Several documents are cited throughout the text of this specification. Each of the documents cited herein (including any manufacturer's specifications, instructions, etc.) are hereby incorporated by reference; however, there is no admission that any document cited is indeed prior art of the present invention. Further incorporated by reference is the complete disclosure content of the co-pending application filed in the name of IDEA AG and bearing the title "Noninvasive vaccination through skin".

Nasal delivery has been explored extensively over the last decades and was discussed repeatedly as an alternative to the systemic delivery of drugs, especially peptides and proteins, which normally must be injected. Nasal delivery also attracted interest owing to the fact that it avoids the hepatic first-pass effect, the problem of degradation in nasal cavity notwithstanding, which creates a pseudo-first-pass effect (Sarkar, 1992). The latter difficulty prompted chemical or recombinant structural peptide or protein modifications to improve the stability and minimise the enzymatic cleavage of macromolecules in the nose (Wearley, 1991).

Some earlier reviewers (Illum, 1991; Wearley, 1991) expected that transnasal peptide delivery, supported by absorption enhancers, will provide a convenient, efficient means for the administration of protein and peptide therapeutics. More recent surveyors took less optimistic stance, however (Harris, 1993). Rapid metabolism and nonlinear pharmacokinetics of nasally delivered peptides (Wearley, 1991) are partly responsible for this. The other reasons are the anatomical and temporal barriers presented by the

nasal mucosa (Sarkar, 1992), and especially the intolerable side effects of most, if not all, methods currently in use for nasal delivery. This holds also true for efforts to deliver compounds with the aim to generate a protective immune response transnasally, which would represent a more natural way of antigen presentation than encountered by conventional injection. The adverse side effects observed with transnasal immunisation experiments are mainly due to the presence of immunoadjuvants (such as Cholera toxin (CT) or its fragment B, heat labile protein from *E. coli*, keyhole limpet hemocyanin, or other substances with ADP-rybosilating activity, for example), and/or molecules with a permeation enhancing activity, in addition to the antigen in the formulation for nasal delivery. While the former may be toxic, the latter are irritating to the immunised subject. Selectivity of immune response, moreover, cannot be achieved with unspecific stimulatory agents. Moreover, there is substantial variability in the resulting immune response after nasal antigen administration, probably due to the difficulty of depositing the immunogen on the sites in the nasal cavity with the lowest transbarrier transport resistance.

The human nasal cavities with a total volume of 15 mL and a total surface area of 150 cm<sup>2</sup> - which amounts to more than 1 m<sup>2</sup> if one allows for the surface corrugations - are covered by mucus and a mucosa 2 mm to 4 mm thick. Most of the cavity surface is lined by a respiratory epithelium, comprised of columnar cells, goblet cells, and ciliary cuboidal cells. The resulting permeability barrier is related to that of the oral cavity, with which it communicates and which is covered by a keratinised barrier tissue. In either case, the cells in the barrier are tightly packed and often sealed with the specialised intercellular lipid arrangements. Moreover, in either case, the permeability barrier is lowered by the topical use of substances which compromise the quality and packing of such lipidic seals and/or which increase the probability for molecular partitioning into the barrier. Deviant from the situation encountered in the mouth, from the nose foreign substances are cleared into the nasopharynx by the cilia, with an average speed of 5 mm/min. An exception is the upper region of nasal cavity, which contains no cilia but is covered by a pseudo-stratified olfactory neuroepithelium. The nasal subepithelium contains a dense vascular network and the venous blood from the nose passes directly into the systemic circulation.

Nasal route of delivery has been relatively unsuccessful to date when used for high molecular weight substances. Use of permeation enhancers did not improve the

situation sufficiently, largely due to the fact that such substances are generally poorly tolerated and of limited usefulness. The pharmacodynamics resulting from nasal drug delivery is also highly variable. Major reasons for this are the inconsistency in the site of deposition or in the delivery details, as well as changes in the mucous secretion and mucociliary clearance; the latter are compounded especially by the presence of allergy, hay fever, and the common cold in treated subjects (Harris, 1993). Protein degradation in mucosa is important as well (Sarkar, 1992). Despite this, numerous studies were done with buserelin, vasopressin, cholecystokinin, calcitonin, growth hormone and related substances (e.g. GHRH), erythropoietin, G-CSF, interferon, insulin, gonadotropin hormone releasing hormones (GnRH), and vasopressin analogues, the results of which are reviewed briefly in the following.

### **Systemic delivery of large drugs through the nose**

Hexarelin (GH analogue; MW  $\approx$  800). The GH response to the intranasal hexarelin administration (about 18  $\mu$ g/kg) was not significantly higher than that induced by an injection of 1  $\mu$ g GHRH/kg (Ghigo et al., 1996). On the other hand, the former kind of treatment did not significantly modify IGF-I but increased IGFBP-3 levels. Both IGF-I and IGFBP-3 levels were slightly but significantly increased by oral treatment with the drug as well (Ghigo et al., 1996).

Intranasal treatment with octreotide nasal powder, a somatostatin analogue (up to 2 mg TID, corresponding to a mean GH value below 5  $\mu$ g/L during 8 daytime hours), was well tolerated, with only mild side effects and no significant changes in the nasal mucosa. An improvement of the clinical picture was registered in all patients after a few days of octreotide nasal powder administration. Positive correlation was found between GH and IGF-I, GH and IGFBP-3, IGF-I and IGFBP-3, insulin and IGFBP-3 and insulin and IGF-I during chronic (3-6 months) treatment (Invitti et al., 1996).

Cholecystokinin (MW  $\approx$  1050). The carboxy terminal octapeptide of cholecystokinin (CCK-8) has similar functions as native cholecystokinin (CCK), but lacks receptor selectivity and metabolic stability. Mediation of satiety via the A-receptor subtype can be used for management of obesity. This was also shown after intranasal administration of Hpa(SO<sub>3</sub>H)-Nle-Gly-Trp-Nle-MeAsp-Phe-NH<sub>2</sub>, the result of moving

the N-methyl group from Phe to Asp, which inhibited feeding in beagle dogs (Pierson et al., 1997).

After intranasal (10  $\mu\text{g}$ ) and intravenous (0.25  $\mu\text{g}$  and 2.5  $\mu\text{g}$ ) administration of an octapeptide derivative of cholecystokinin, the substance CCK-8 was shown to affect the auditory event related potential (AERP) in 20 healthy subjects. The effect was stronger in women than in men (Pietrowsky et al., 1996). Plasma CCK-8 concentrations after intranasal administration of 10  $\mu\text{g}$  CCK-8 were comparable to those of 0.25  $\mu\text{g}$  CCK-8 given i.v., but were substantially lower than those elicited by 2.5  $\mu\text{g}$  CCK-8 (Pietrowsky et al., 1996).

Vasopressin (MW = 1054). Vasopressin DGAVP (2 mg) was administered intranasally and orally to healthy subjects for 1 week. Peak levels were always observed at 15 min. The mean absorption and elimination half-life (around 8 min and 35-38 min, respectively) were similar for the two tested routes of administration, but the latter only had 0.7 % relative bioavailability (Westenberg et al., 1994).

In a double-blind, crossover study, subjects received on three different occasions 20 IU of (arginine)vasopressin (AVP) intranasally (IN), or 1.5 IU of AVP and saline solution i.v. Evoked potentials (ERPs) were recorded during the subject's performance on a auditory attention task. Plasma concentrations of vasopressin during task performance were enhanced after AVP, with the increase after i.v. administration of AVP exceeding 2000-fold that after AVP i.v. Intranasal administration of AVP substantially increased the P3 component of the ERP in contrast to the injection (Pietrowsky et al., 1996).

Acute (2 mg) and chronic, 2 weeks treatment (1 mg/day) with nasal DGAVP revealed an improved short-term memory for abstract words in males but not in females, with no positive effect on learning concrete words. Chronic, but not acute, treatment with DGAVP reduced the reaction time for scanning of digits in a memory comparison task (Sternberg paradigm) in both sexes (Bruins et al., 1995). In a different human study, arginine-vasopressin (AVP: 3x10 IU) enhanced memory performance after nasal administration. The late positive complex (LPC) elicited by oddball stimuli was not affected whereas the structural encoding task revealed an effect of the drug. In both studies, AVP intake resulted in a marked change of the scalp distribution of the P3 component, which is a prominent part of the LPC. Vasopressin was thus concluded to



To simulate endogenous production of growth hormone the protein was administered on three occasions intranasally in doses of 0.05, 0.10 and 0.20 IU/kg, using didecanoyl-L- $\alpha$ -phosphatidylcholine as an enhancer (Laurson et al., 1996). On the other two occasions the patients received an s.c. injection (0.10 IU/kg) and an i.v. injection (0.015 IU/kg) of GH, respectively. The nasal doses and the s.c. injection were given in random order in a crossover design. Intravenous administration produced a short-lived serum GH peak value of 128  $\mu$ g/L. Peak levels were around 14  $\mu$ g/L after s.c. injection (50% bioavailability) and between 3  $\mu$ g/L and 8  $\mu$ g/L, respectively, after the three nasal doses (bioavailability between 4% and 9%). Serum insulin-like growth factor I (IGF- I) levels increased significantly after s.c. administration only. However, the data revealed that a closer imitation of the physiological GH pulses was achieved via the nose. Despite this the authors of the study concluded that GH administration is of limited importance for the induction of a metabolic response to GH (Laurson et al., 1996).

GHRP-2 is one of the most potent members of the GHRP family, which exerts its biological activity after oral, intranasal and i.v. administration. For example, the children who had a robust response to the injected GH-releasing factors also received intranasal GHRP-2, with significant, but not quantitated, response over a dose range of 5-20  $\mu$ g/kg per dose (Pihoker et al., 1995).

Insulin (MW = 5808). The problem of low bioavailability of insulin solutions given through the nasal mucosa was improved by using absorption enhancers or bioadhesive microspheres (Gizurason & Bechgaard, 1991; Illum & Davis, 1992). Bioavailability greater than 10% was measured but to date no corresponding formulation has found its way into the late clinical trials. The chief reason for this appears to be the severe damage to nasal mucosa caused by the commonly used permeation enhancers.

For example, following the administration of powder formulations comprising insulin and the permeation enhancer sodium tauro-24,25-dihydrofusidate (STDHF), the hypoglycaemic response and the serum insulin levels in sheep increased with STDHF/insulin molar ratio in the range 0 to 16.8 (Lee et al., 1991). The reason for this is increased mucosal permeability as well as reduced insulin aggregate size. The bioavailability ranges from 2.9% to 37.8% for the powder, and was reported to be



15.7% and 37.4%, respectively for the drops or spray containing STDHF/insulin = 8.4/1 mixture, and roughly proportionally to the enhancer concentration (Lee et al., 1991). To achieve a high bioavailability major changes in nasal mucosa had to be tolerated, however.

In humans, the 200 U insulin/mL formulation containing a blend of enhancers (didecanoyl-phosphatidylcholine (2 w-%), glycerol (1.6 w-%), 0.4 w-% fractionated coconut oil) and 0.2 w-% cholesterol resulted in appr. 8 % bioavailability, the highest values having been measured for the high dose (2x3 sprays of 50  $\mu$ L each), which also was most irritant (Drejer et al., 1991).

Cyclodextrins dissociate insulin hexamers into smaller aggregates, in dependence on structure and concentration. Hexamer dissociation was therefore speculated to be the reason for higher nasal absorption of the polypeptide (Shao et al., 1992). The relative effectiveness of various cyclodextrins for this purpose was reported to decrease from dimethyl- $\beta$ -cyclodextrin (DM- $\beta$ -CD) >  $\alpha$ -cyclodextrin ( $\alpha$ -CD) >  $\beta$ -cyclodextrin ( $\beta$ -CD), hydroxypropyl- $\beta$ -cyclodextrin (HP- $\beta$ -CD) >  $\gamma$ -cyclodextrin (gamma-CD). A direct relationship between absorption promotion and nasal membrane protein and lipid release was invoked to explain such sequence (Shao et al., 1992).

It is less clear why cationic chitosan enhances the absorption of insulin across the nasal mucosa of rat and sheep in a concentration dependent fashion, with optimum concentrations higher than 0.2% and 0.5% in rats and sheep, respectively, but overall efficiency of this procedure is only around 10% (Illum et al., 1994). Using didecanoyl-L- $\alpha$ -phosphatidylcholine as an enhancer results in 4% to 9% of nasal insulin bioavailability (Laursen et al., 1996).

G-CSF (MW = 19600). The relative bioavailability of rhG-CSF administered nasally in the rat was approximately 2%, compared to an s.c. injection, as evaluated from the immunologically active rhG-CSF concentration in rat plasma and the area under the curve (AUC) at t=8 h. Leukocyte stimulation counts suggested 5-10% availability at t=48 h. Relative bioavailability and pharmacological availability were increased 23 times and 3 times, respectively, by polyoxyethylene 9-lauryl ether (Laureth-9), but no increase in availability occurred with sodium glycocholate (Machida et al., 1993).

Absorption of dissolved recombinant human granulocyte colony-stimulating factors (rhG-CSF at pH 4) through the nose of rabbits was investigated with dimethyl- $\beta$ -cyclodextrin added or without such excipient, which acts as barrier permeation enhancer. The proteins were absorbed and the total leukocyte numbers in peripheral blood increased in either case, but excipients improved the absorption of rhG-CSF appreciably (Watanabe et al., 1993). A subsequent pharmacokinetic and pharmacodynamic study (Watanabe et al., 1995) revealed that protein is absorbed through the nasal cavity from a solution, especially in the presence of alpha-cyclodextrin ( $\alpha$ -CyD), which can act as carrier in the membrane. Good correlation was found between the logarithm of the area under the serum G-CSF concentration-time curve (AUC) and the area under the increased total blood leukocyte count-time curve (Watanabe et al., 1995).

Interferon (MW = 23000). Treatment of experimental rhinovirus colds in 38 adults by intranasal administration of recombinant interferon beta serine (MW = 18500) had no effect on illness rate or severity, but did decrease the frequency of virus shedding by the factor of 2 (on day 4) to 3 (on day 6). The course of middle-ear dysfunction associated with experimental colds was also positively affected by the drug (Sperber et al., 1992).

Erythropoietin (MW = 30400). The pharmacological availability of rh-EPO after intranasal administration without enhancers was compared to that of intravenous injections. The pharmacological activity was enhanced in low pH and hypotonic mannitol solution, which both compromise the barrier quality. This resulted in relative bioavailability of nasally applied drug between 7% and 4%, when estimated by different reticulocyte counting methods. (Shimoda et al., 1995).

Labelled dextrane (MW = 4100, 9000, 17500), applied nasally at the dose of 6.5 mg, was seen to pass mucosa in the presence of glycocholate (3 mg) and found in the blood in concentration range between 6 ng/mL and 21 ng/mL, which corresponds to app. 0.05%, 0.02%, and 0.01% for the three molecular sizes, respectively (Maitani et al., 1989).



nature of the particles, their capacity to adsorb antigens, and/or their size seem to play only a minor role in the process (Maejima et al., 1997).

Hollow spheres, according to Ting et al. (1992), are unsuitable for nasal delivery, owing to their rapid clearance and variable deposition pattern. Polyvinyl alcohol microparticles in the form of collapsed, solid spheres with the desired size for nasal deposition (10-200  $\mu\text{m}$ ) were therefore produced by spray-drying and spray-desolvation (Ting et al., 1992).

The above observation notwithstanding, several kinds of particulate suspensions were used in the nose, typically to elicit antibodies against the particle-associated antigens.

This includes so-called proteosomes comprising gp160 (Lowell et al., 1997) or influenza virus proteins. Another example are particles made from polymerised carbohydrates coated with a lipid (bi)layer.

It is important to realise, however, that in any nasal uptake study one should consider and allow for secondary redistribution. For example, the biodistribution of radioactivity from the purified major *Parietaria judaica* allergen after sublingual, oral, and intranasal administration in healthy human volunteers is similar. This is indicative of test material swallowing and absorption in the gastrointestinal tract (Bagnasco et al., 1997). In the intranasal case, transport to the pharynx by mucociliary clearance plays an important role as well, but a relevant fraction of the tracer is retained on the nasal mucosa for up to 48 hours after administration (Bagnasco et al., 1997).

### **Oral spill-over and the danger of false positive results**

Proteins are absorbed in the gastrointestinal tract, albeit in small quantities. For example, ovalbumin (OVA) is absorbed in the stomach as well as from the GI tract into the blood and lymph circulation at levels of 0.007-0.008% and 0.0007-0.002% of applied dose; a higher dose in the latter case leads to relatively higher absorption (Tsume et al., 1996). Stomach absorption supplies nearly exclusively the blood, suggesting different mechanisms and/or routes of absorption between the stomach and the small intestine. OVA association with liposomes can improve the uptake about 2 to 3-fold, possibly owing to slower enzymatic degradation of OVA.

Often, the result of nasal and oral immunisation are very similar, suggesting that part of the effect of the former may be due to the spill over of the antigen into gastro-intestinal tract. Data obtained with human adenovirus type 5, used as a vector for heterologous DNA sequences, illustrate this (Flanagan et al., 1997).

### **Transnasal delivery into the central nervous tissue (CNS)**

The access of substances to the brain is of paramount importance for the treatment of psychiatric and neurologic diseases. Transnasal route of delivery into the CNS was therefore tested for a few selected bioactive molecules.

To date, drug delivery into the CNS tissue by nasal administration has received little attention (Pesechnik & Price, 1996). Wheat-germ agglutinin coupled to horseradish peroxidase was demonstrated to be taken up by the cells of olfactory nerve, resulting in concentration in the olfactory bulb around 0.1% of applied concentration; the underlying principle is probably receptor-mediated endocytosis of WGA and subsequent trans-synaptic, retrograde transfer towards the brain. A similar mechanism is also possible in the case of viral infections in the nose.

For example, an intranasal instillation of vesicular stomatitis virus (VSV), a negative-sense RNA virus, may result in a lethal infection of murine and rat brain (Huneycutt et al, 1994). Within 12 h following intranasal inoculation of VSV, this antigen can be detected in the olfactory nerve layer of the ipsilateral olfactory bulb. Within 3-4 days post-inoculation (p.i.), VSV had disseminated into the glomeruli of the olfactory bulb as well as the anterior olfactory nuclei, ipsilateral to the VSV instillation. Within the glomeruli, VSV antigen is more prevalent in the granule cells than in the mitral cells. Correspondingly, the lateral olfactory tract, where axons of mitral cells course, remain VSV negative throughout 7 days p.i. By 7 days p.i., viral proteins are detected in several additional regions extending to the brainstem. The pattern of VSV immunoreactivity supports the picture of initial infection of the olfactory bulb glomeruli, with subsequent spreads via both ventricular surfaces and retrograde transport within axons of neuromodulatory transmitter systems enervating the olfactory bulb (Huneycutt et al, 1994).

Draghia et al. (1995) have demonstrated that it is possible to transfer the *Escherichia coli* lacZ gene in vivo into the central nervous system structures of rats after nasal instillation of replication-defective adenoviral vector AdRSV beta gal. Mitral cells from the olfactory bulb, neurons from the anterior olfactory nucleus, locus coeruleus and area postrema expressed beta-galactosidase for at least 12 days (Draghia et al., 1995). Parainfluenza type 1 vaccine virus also directly accesses the central nervous system by infecting olfactory neurons (Mori et al., 1996).

However, it would be highly desirable to have a convenient and reliable transnasal transport system for the compounds that are capable of and intended to generate a protective immune response without simultaneously generating a variety of adverse side effects. Common types of non-invasive applications, including oral immunisation, often do not elicit the desired immune response. Many injectible vaccines also do not provide optimum antibody isotype pattern, mainly due to the unnatural route of antigen entry into the body. Transnasal immunisation remains problematic owing to the large size of typical immunogen which is subject to similar restrictions as the transport of pharmaceutically active compounds across the nasal mucosa.

In conclusion, although the prior art has tested various approaches to transnasal delivery it has hitherto failed to provide a convincing principle for convenient and well tolerated transfer of compounds, such as pharmaceutically active substances, immunogens/antigens or allergens, through the nasal barrier, in particular if said compounds are large. The solution to said technical problem, i.e. the provision of a suitable system, is provided by the embodiments characterised in the claims.

Accordingly, the present invention relates to use of a penetrant, suspended or dispersed in a solvent, in the form of a minute fluid droplet surrounded by a membrane-like coating of one or several layers of at least two different substances or two different forms of a substance with the tendency to aggregate, said substances or forms of a substance differing by at least the factor of 10 in solubility in a preferably aqueous liquid medium, such that the average diameter of homo-aggregates of the more soluble substance or form of the substance or the average diameter of the hetero-aggregates consisting of both said substances or forms of said substance is smaller than the average diameter of homo-aggregates of the less soluble substance or form of the substance and/or wherein the more soluble component tends to solubilise the

penetrating droplet and wherein the content of such component amounts to up to 99 mol-% of the concentration required to solubilise the droplet or else corresponds to up to 99 mol-% of the saturating concentration in the un-solubilised droplet, whichever is higher, and/or wherein the elastic deformation energy of the droplet surrounding the membrane-like coating is at least 5x lower, more preferably is at least 10x lower and ideally is more than 10x lower than that of the red blood cells or of the phospholipid bilayers with fluid aliphatic chains, such droplets then acting as carriers, for the transnasal administration of pharmaceutically active compounds, antigens, allergens, mixture of antigens and/or mixture of allergens.

These compounds, antigens or allergens do not cross the nasal mucosa in a practically meaningful quantity on their own without causing unacceptable side effects.

As regards the above recited values of up to 99%, it is to be noted that values below 50% of the former relative concentration are particularly useful, with values below 40 rel-% or even around and below 30 rel-% being even more advantageous, whereas in the case of droplets which cannot be solubilised by the more soluble component relative concentrations which exceed the above mentioned relative concentrations by the factor of up to 2 are most preferred.

Formulations including the above-referenced penetrants are described in detail in DE 41 07 152, PCT/EP91/01596, PCT/EP96/04526, and DE 44 47 287, which are incorporated herewith by reference. Relevant information useful for penetrant manufacturing and loading with various macromolecular actives, which are too big to permeate through the barrier, is given in patent application PCT/EP98/06750, also incorporated herewith by reference.

More general information on lipid suspensions can be found in the handbook dealing with 'Liposomes' (Gregoriadis, G., ed., CRC Press, Boca Raton, FL, Vols 1-3, 1987), in the book 'Liposomes as drug carriers' (Gregoriadis, G., ed., John Wiley & Sons, New York, 1988), or in the laboratory manual 'Liposomes. A Practical Approach' (New, R., Oxford-Press, 1989). The properties of phospholipids, which can be used conveniently to prepare bio-compatible immunopenetrants, are reviewed in 'Phospholipids Handbook' (Cevc, G., ed., Dekker, New York, 1995).

The manufacturing temperature for said penetrants is normally chosen in the 0°C to 95°C range. Preferably, one works in the temperature range 10-70°C, most frequently at temperatures between 15°C and 45°C, under all circumstances below the temperature at which any important formulation ingredient would undergo an irreversible change in the composition or physical state. These temperatures can be determined by the person skilled in the art using his common general knowledge and the teachings of the various documents cited in this specification. (For reference: the skin temperature is normally around 32°C.) Other temperature ranges are possible, most notably for the systems containing freezable or non-volatile components, cryo- or heat-stabilised formulations, etc.

If required to maintain the integrity and the desired properties of individual system components, carrier formulations can be stored in the cold (e.g. at 4°C), with or without associated active agents. It is also possible, and sometimes sensible, to manufacture and store the preparation under an inert atmosphere, e.g. under nitrogen. The shelf-life of carrier formulation, moreover, can be extended by using substances with a small number of double bonds, that is, by a low degree of unsaturation, by choosing peroxide-arm ingredients, by including antioxidants, chelators, and other stabilising agents, or by preparing the agent loaded penetrants *ad hoc* or *in situ*, e.g. from a freeze dried or dry mixture.

The term "two forms of a substance" in connection with this invention means two ionization states or salt forms of the same substance, two different complexes of such substance, etc.

"Non-invasive administration" or "non-invasive delivery" in this specification denotes application on or transport through the nasal mucosa.

"Nasal administration", in the context of this document, refers to applications of test material, whether by direct intranasal intubation, spontaneous sniff of a drop of the test fluid, or an inhalation of the sprayed test-fluid into the nose, independent of precise site of impact or deposition.

The term "penetration" in this application describes non-diffusive motion of large entities across a barrier. This process is believed to involve penetrant adaptation to the



otherwise confining pores in the barrier, perhaps in association with a transient, selective, and reversible decrease in the barrier resistance.

The term "permeation" refers to a diffusion across the semipermeable barrier and is typically driven by the permeant concentration gradient across the barrier.

A penetrant, consequently, is an entity comprising a single molecule or an arrangement of molecules too big to permeate through a barrier but capable to cross the barrier owing to the penetrants adaptability to the shape and/or diameter of the otherwise confining passages (pores) of a barrier. This adaptability is seen from the fact, for example, that penetrants more than twice bigger than the pore diameter will cross the bilayer without being fragmented down to the pore size. A permeant, on the other hand, is an entity that can permeate through the semi-permeable barrier, such as the skin. A penetrant in an external field experiences a driving force proportional to the nominal penetrant size and to the applied field, which may occur naturally. Such a force, which on the intact, non-occluded skin is believed to originate from the water concentration gradient across the stratum corneum, can result in a penetrant motion through the barrier, including the skin, if the force is strong enough either to deform the penetrant or else to widen the passages in the barrier sufficiently to elude the problem of size exclusion, or both.

A permeant, on the other hand, is a molecule diffusing, or at least capable of diffusion, across the semi-permeable barrier.

The above-referenced penetrant is typically an ultra-adaptable entity comprising several components. Said penetrant, in the widest sense of the word, is a supra-macromolecular body that can pass spontaneously through the permeability barrier with pores much smaller than the penetrant diameter, and thus transport material from the application to the destination site on either side of the barrier. In order to meet this goal, the penetrant must adjust its properties, most notably its deformability, to the shape and size of the pores in a barrier. This typically occurs under the influence of a strong driving force or a pressure acting on all molecules in the penetrant. Gradients which do not depend on the penetrant concentration, such as hydration or external electric potential difference across the barrier, were shown to serve this purpose.

Lipid aggregates in (quasi)metastable state, and of the nature described above in connection with the invention, most often behave as highly adaptable penetrants, especially when they have the form of a tiny droplet surrounded by one or a few membranes (bilayers) (Cevc et al., 1997; Cevc et al., 1998). Owing to membrane metastability, unusually high local bilayer curvature can develop at the sites of transient, local membrane destabilisation without compromising the overall aggregate integrity. From the composition point of view, such ultra-adaptable and self-regulating vesicles typically consist of a suitably chosen lipid mixture. In order to change conventional lipid vesicles, liposomes, into the optimised vesicles (Transfersomes) one can add, for example, suitable edge-activators into the aggregate membrane (Cevc et al., 1998). Alternatively, molecules which change the system deformability after complexation with or binding to the basic aggregate ingredient can be used. Often, but not necessarily, the activators belong to the class of surfactants below the saturation or solubilization concentration, which in the latter case gives rise to mixed micelles formation. This is important as solubilised lipids, in the form of mixed lipid micelles, can cross the pores sufficiently wider than the micelle diameter but are incapable of enforcing channel opening in the biological tissues, which can be widened and trespassed by the mixed lipid vesicles, however. The postulated reason for this – to which the applicant does not wish to be bound - is the much greater aggregation number of the latter kind of aggregate which translates into the greater sensitivity to external, transport-driving gradients, such as the water activity gradient, and which is then capable of paying the energetic price for the pore or channel opening in the barrier.

The present invention is, in view of the prior art, particularly surprising since ultradeformable lipid vesicles would seem unsuitable for the purpose of transnasal delivery taken that they were reported to date to cross barriers, such as skin, only under non-occlusive conditions, that is, in the presence of a strong trans-barrier water concentration gradient (Cevc et al. 1995; Paul and Cevc, 1995), which is believed not to exist in the strongly hydrated nasal mucosa.

It was unexpectedly found that macromolecules in association with highly adaptable penetrants, typically in the form of mixed lipid vesicles, are transported across nasal mucosa despite the high water content in this mucosa and in the exhaled air saturated with humidity. Concluding from the fact that several successfully tested formulations of



conventional nasal vaccinations in addition to the safety and robustness of administration. Improved safety would reflect the choice of the non-toxic and non-irritating carrier ingredients. Better reproducibility could result from the greater ability of the specially designed carriers, compared to that of the antigens or immunoadjuvants used alone to overcome the nose barrier. Taken the expectation that different carrier populations loaded with the individual antigens could be combined into a final multi-valent vaccine formulation the capability of invented technology to meet the trend in immunotherapy is given.

It stands to reason that non-toxic and „gentle“ formulations containing merely bio-compatible or natural, body-like ingredients, which protect the body faster and/or better than the corresponding antigen injections, would be preferred to the latter and would have a substantial commercial value.

In accordance with the present invention it is recommended to choose the penetrant characteristics, especially the deformability, concentration, or composition of the mixed lipid aggregates, so as to control the rate or the efficiency of penetrant-mediated transport.

In the process of optimisation of the formulation and/or administration it may be convenient to determine the flux of drug or agent loaded penetrants through the pores in a well-defined barrier as a function of suitable driving force or pressure, which act across the barrier, and then to describe the data by a convenient characteristic curve which, in turn, is employed to optimise the formulation or application further.

The pharmaceutically acceptable form of the agent may be given in a variety of final formulations, optionally, and depending on the purpose of the administration, in combination with diverse secondary agents. Such agents will be explained in more detail later in the text and may be, for example, bacterial compounds or other immunomodulations.

Furthermore, the present invention relates to the use of a penetrant, suspended or dispersed in a solvent, in the form of a minute fluid droplet surrounded by a membrane-like coating of one or several layers of at least two different substances or two different forms of a substance with the tendency to aggregate, said substances or forms of a





substances belong to this group. On the other hand, nearly any part of the human body, if incorrectly processed by or exposed to the body's immune system, can result in an auto-immune response and lead to the allergic reaction to such a substance. In the narrower interpretation, used when so stated, an allergen is a substance, a group, or an arrangement of substances causing immediate hypersensitivity reactions in the body that could be diminished, or even eliminated, by an immunotherapy, whether done non-invasively through the nasal mucosa or not.

An „antigen“ is a part of a pathogen or an allergen in its natural form or after fragmentation or derivatisation. More generally, the word antigen denotes a macromolecule or a fragment thereof, any haptenic moiety (for example, a simple carbohydrate, complex carbohydrate, polysaccharide, deoxyribonucleic acid), in short, any molecule recognized by a body's antibody repertoire and possibly capable of antibody induction when administered in the system. A macromolecular antigen is defined as an antigen that is known to or believed to cross spontaneously the nasal barrier only in quantity too small for the desired practical purpose. Thus, macromolecules are molecules that, on their own, do not cross the nasal mucosa in practically useful quantity without causing unacceptable side effects.

The term "a mixture of antigens" or "a mixture of allergens" means, in accordance with the present invention, the combination of at least two antigens and/or allergens. It is envisaged that also mixtures of antigens and allergens can be used according to the present invention.

Furthermore, the present invention relates to a pharmaceutical composition for transnasal administration comprising a carrier which is a penetrant, suspended or dispersed in a solvent, in the form of a minute fluid droplet surrounded by a membrane-like coating of one or several layers of at least two different substances or two different forms of a substance with the tendency to aggregate, said substances or forms of a substance differing by at least the factor of 10 in solubility in a preferably aqueous, liquid medium, such that the average diameter of homo-aggregates of the more soluble substance or form of the substance or the average diameter of the hetero-aggregates consisting of both said substances or forms of said substance is smaller than the average diameter of homo-aggregates of the less soluble substance or form of the substance and/or wherein the more soluble component tends to solubilise the

penetrating droplet and wherein the content of such component amounts to up to 99 mol-% of the concentration required to solubilise the droplet or else corresponds to up to 99 mol-% of the saturating concentration in the un-solubilised droplet, whichever is higher, and/or wherein the elastic deformation energy of the droplet surrounding the membrane-like coating is at least 5x lower, more preferably is at least 10x lower and ideally is more than 10x lower than that of the red blood cells or of the phospholipid bilayers with fluid aliphatic chains and a pharmaceutically active ingredient.

In a preferred embodiment of the use or the pharmaceutical composition of the present invention the pharmaceutically active ingredient is an adrenocorticostaticum, an adrenolyticum, an androgen or antiandrogen, an antiparasiticum, an anabolicum, an anaestheticum or analgesicum, an analepticum, an antiallergicum, antiarrhythmicum, antiarteroscleroticum, antiasthmaticum and/or bronchospasmolyticum, an antibioticum, an anti-infective agent, an antidepressivum and/or antipsychoticum, an antidiabeticum, an antidot, an antiemeticum, antiepilepticum, antifibrinolyticum, anticonvulsivum or anticholinergicum, an enzyme, a coenzyme or the corresponding enzyme inhibitor, an antihistaminicum (and combinations thereof) or antihypertonicum, an antihypotonicum, an anticoagulant, antimycoticum, antimyasthenicum, an agent against Morbus Alzheimer or Morbus Parkinson, an agent for ACS therapy, an antiphlogisticum, antipyreticum, antirheumaticum, antisepticum, a respiratory analepticum or a respiratory stimulant, a broncholyticum, cardi tonicum, chemotherapeuticum, a coronary dilatator, a cytostaticum, a diureticum, a ganglium-blocker, a glucocorticoid, an anti-flew agent, a haemostaticum, hypnoticum, an immunoglobuline or its fragment or any other immunologically active substance, such as an immunomodulator, a bioactive carbohydrate (derivative), a contraceptive, an anti-migraine agent, a corticosteroid, a muscle relaxant, a narcoticum, a neurotherapeuticum, a (poly)nucleotide, a neurolepticum, a neurotransmitter, a (poly)peptide (derivative), an opiate, an ophthalmicum, (para)-sympaticomimeticum or (para)sympathicolyticum, a protein(derivative), a psoriasis/neurodermitis drug, a mydriaticum, a psychostimulant, rhinologicum, a sleep-inducing agent, a sedating agent, a spasmolyticum, tuberculostaticum, an urologicum, a vasoconstrictor or vasodilatator, a virustaticum, a wound-healing substance, an alcohol abuse preparation, an anticonvulsant, an antineoplastic, an antirheumatic, an appetite suppressant, a biological response modifier, a blood modifier, a bone metabolism regulator, a cardioprotective agent, a cardiovascular agent, a central nervous system stimulant, an enzyme, an agent for





*Bacillus pestis*, BCG), particularly anaerobes, such as the *Clostridium* species (e.g. *Clostridium tetani*, *Clostridium perfringens*, *Clostridium novyi*, *Clostridium septicum*), bacteria and viruses, which survive and replicate within host cells, comprising mycobacteria (e.g. *M. tuberculosis*) and *Listeria monocytogenes*, retro- and adenoviruses, including hepatitis virus, (human) immunodeficiency virus, herpes viruses, small-pox (chicken-pox), influenza, measles, mumps and polio viruses, cytomegalovirus, rhinovirus, etc., and fungi prospering inside host cells, parasites including animal parasites, such as protozoa and helminths, and ectoparasites, such as ticks and mites, or *Brucella* species (e.g. *B. melitensis*, *B. abortus*, *B. suis*, *B. canis*, *B. neotomae*, *B. ovis*), the causative agent for cholera (e.g. *Vibrio cholerae*), *Haemophilus* species like *H. actinomycentemcomitans*, *H. pleuropneumoniae*, as well as pathogens triggering paratyphoid, plague, rabies, tetanus and rubella diseases; eukaryotic cells or their parts that cause various neoplasiae, auto-immune diseases and other pathological states of the animal or human body, which do not necessarily result from microbial infections, also belong in this group.

It is most preferred that the antigen, preferably the pathogen, is used in a purified, or even better in a pure form.

Pathogens causing major infective diseases such as hepatitis virus, (human) immunodeficiency virus, herpes viruses, small-pox (chicken-pox), influenza, measles, mumps and polio viruses, cytomegalovirus, rhinovirus, etc., and fungi prospering inside host cells, a parasite including animal parasites, such as protozoa and helminths, and ectoparasites, such as ticks and mites, or *Brucella* species, or the causative agent for cholera, *Haemophilus* species, as well as pathogens triggering paratyphoid, plague, rabies, tetanus and rubella diseases are particularly preferred as are eukaryotic cells or their parts that cause various neoplasiae, auto-immune diseases and other pathological states of the animal or human body, which do not necessarily result from microbial infections.

In another preferred embodiment of the use or the pharmaceutical composition of the invention the allergen is of xenogenic or endogenic origin, derived from a microorganism, an animal or a plant, or belonging to the group of man made and/or irritating inorganic substances, or to such parts or components of the human body which were incorrectly processed by or exposed to the body immune system.

In further preferred embodiment of the use or the pharmaceutical composition of the present invention the allergen belongs to the class of the inhalation allergens, including but not limited to various pollen, spores, bits of animal hair, skin, feather, natural and synthetic textiles, wheat, (house) dust, including mite; furthermore, food and drug allergens; contact allergens; injection, invasion or depot allergens, such as various (gastrointestine-resident) worms, echinococci, trichines, etc., or is a part of implantation material.

In an additional preferred embodiment of the use or the pharmaceutical composition of the present invention said pharmaceutical composition comprises a compound which releases or induces cytokine or anti-cytokine activity or exerts such an activity itself.

The term „cytokine“, as used in the present invention, denotes cytokines, such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, with all subtypes, such as IL-1 $\alpha$  and IL-1 $\beta$ , tumour necrosis factor (TNF), transforming growth factor (TGF- $\beta$  and - $\alpha$ ), Type I and II interferons (IFN- $\alpha$ 1, IFN- $\alpha$ 2, (IFN- $\omega$ ), IFN- $\beta$ , IFN- $\gamma$ ), migration inhibitory factor, MIF, c-kit ligand, granulocyte macrophage colony stimulating factor (GM-CSF), monocyte macrophage colony stimulating factor (M-CSF), granulocyte colony stimulating factor (G-CSF), chemokines, etc., as well as all functional derivatives of any of these molecules.

Cytokines that mediate natural immunity particularly well include type I interferons (IFN- $\alpha$  and IFN- $\beta$ ), tumour necrosis factor (TNF), interleukin-1 (IL-1 $\alpha$  and IL-1 $\beta$ ), interleukin-6 (IL-6) and leukocytes attracting and activating chemokines. Antiproliferative (e.g. with IFN-s), pro-inflammatory (e.g. with TNF, IL-1) or co-stimulatory (e.g. with IL-6) action, amongst other, may be generated by transnasal administration of the pharmaceutical composition described in accordance with the present invention. Cytokines which best mediate lymphocyte activation, growth and differentiation include interleukin 2 (IL-2), interleukin-4 (IL-4) and transforming growth factor (TGF). Such cytokines, consequently, not only can affect target growth but, moreover, influence the activation of, and thus the production of other cytokines by, the cells which finally may play a role in therapeutic or prophylactic action.



where  $R_1$  and  $R_2$  is an aliphatic chain, typically a  $C_{10-20}$ -acyl, or -alkyl or a partly unsaturated fatty acid residue, in particular, an oleoyl-, palmitoeloyl-, elaidoyl-, linoleyl-, linolenyl-, linolenoyl-, arachidoyl-, vaccinyl-, lauroyl-, myristoyl-, palmitoyl-, or stearoyl chain, and where  $R_3$  is hydrogen, 2-trimethylamino-1-ethyl, 2-amino-1-ethyl,  $C_{1-4}$ -alkyl,  $C_{1-5}$ -alkyl substituted with carboxy,  $C_{2-5}$ -alkyl substituted with hydroxy,  $C_{2-5}$ -alkyl substituted with carboxy and hydroxy, or  $C_{2-5}$ -alkyl substituted with carboxy and amino, inositol, sphingosine, or salts of said substances, said lipid compound also glycerides, isoprenoid lipids, steroids, sterines or sterols, of sulphur- or calcium containing lipids, or any other bilayer forming lipids, and preferably is selected from the group of phosphatidylcholines, phosphatidylethanolamines, phosphatidylserines, phosphatidylinositols, phosphatidic acids, phosphatidylserines, sphingosine, and other sphingophospholipids, glycosphingolipids (including ceramidepolyhexosides, sulphatides, sphingoplasmalogens), gangliosides, glycolipids or synthetic lipids, in particular with corresponding sphingosine derivatives, or any other glycolipids, whereby two similar or different chains can be esterified to the backbone (as in diacyl and dialkenoyl compound) or be attached to the backbone with ether bonds, as in dialkyl-lipids, or belong to the backbone as in sphingolipids.

The surfactant used, normally, is nonionic, zwitterionic, anionic or cationic, especially a fatty-acid or -alcohol, an alkyl-tri/di/methyl-ammonium salt, an alkylsulphate salt, a monovalent salt of cholate, deoxycholate, glycocholate, glycodeoxycholate, taurodeoxycholate, taurocholate, etc., an acyl- or alkanoyl-dimethyl-aminoxide, esp. a dodecyl- dimethyl-aminoxide, an alkyl- or alkanoyl-N-methylglucamide, N-alkyl-N,N-dimethylglycine, 3-(acyldimethylammonio)-alkanesulphonate, N-acyl-sulphobetaine, a polyethylene-glycol-octylphenyl ether, esp. a nonaethylene-glycol-octylphenyl ether, a polyethylene-acyl ether, esp. a nonaethylen-dodecyl ether, a polyethylene-glycol-isoacyl ether, esp. a octaethylene-glycol-isotridecyl ether, polyethylene-acyl ether, esp. octaethylenedodecyl ether, polyethylene-glycol-sorbitane-acyl ester, such as polyethylenglykol-20-monolaurate (Tween 20) or polyethylenglykol-20-sorbitanmonooleate (Tween 80), a polyhydroxyethylene-acyl ether, esp. polyhydroxyethylene-lauryl-, -myristoyl-, -cetylstearyl-, or -oleoyl ether, as in polyhydroxyethylene-4 or 6 or 8 or 10 or 12, etc., -lauryl ether (as in Brij series), or in the corresponding ester, e.g. of polyhydroxyethylen-8-stearate (Myrj 45), myristate-, -laurate, linoleate-, linolenate-, palmitoleate- or -oleate type, or in polyethoxylated castor oil 40, a sorbitane-monoalkylate (e.g. in Arlacel or Span), esp. sorbitane-monolaurate, -myristate,

-linoleate, -linolenate-, -palmitoleate- or -oleate, an acyl- or alkanoyl-N-methylglucamide, esp. in or decanoyl- or dodecanoyl-N-methylglucamide, an alkylsulphate (salt), e.g. in lauryl-, myristoyl, palmitoyl, oleoyl-, palmitoleoyl-, linolenyl-, linoleoyl-, vaccinyl-, or elaidoyl-sulphate, sodium deoxycholate, sodium glycodeoxycholate, sodium oleate, sodium taurate, a fatty acid salt, with similar preference for aliphatic chains as given above, a lysophospholipid, such as n-octadecylene(=oleoyl)-glycerophosphatidic acid, -phosphorylglycerol, or -phosphorylserine, n-acyl-, e.g. lauryl, myristoyl, palmitoyl, oleoyl-, palmitoleoyl-, elaidyl-, vaccinyl-, linoleyl-, linolenyl-glycero-phosphatidic acid, -phosphorylglycerol, or -phosphorylserine, or a corresponding short, double chain phospholipid, such dodecylphosphatidylcholine, or else is a surface-active polypeptide. It is important to realise, however, that complexes of polar lipids with other amphipats often can take the role of surfactants in the coating of a carrier and that different ionisation or salt forms of the polar lipids may differ widely in their properties. It therefore stands to reason that two different physicochemical states of the same (polar) lipid mixed together in a membrane may produce a highly deformable carrier satisfying the conditions of this invention.

In an additional preferred embodiment of the use or of the pharmaceutical composition of the present invention, the more soluble component is an agent to be transported across the barrier, said agent having a tendency to form common large structures with the less soluble component(s) of the penetrant, typically in the form of a physical or a chemical complex.

In a further preferred use or pharmaceutical composition of the invention, the more soluble component tends to solubilise the penetrating droplet and is present in concentration not exceeding 99 mol% of the concentration required to disintegrate the droplet or, alternatively, not exceeding 99 mol% of the saturating concentration in the unsolubilised droplet, whichever is higher, values below 50% of the former relative concentration being particularly useful, with values below 40 rel-% or even around and below 30 rel-% being even more advantageous, whereas in the case of droplets which cannot be solubilised by the more soluble component relative concentrations which exceed the above mentioned relative concentrations by the factor of up to 2 are most preferred.

In a different preferred embodiment of the use or of the pharmaceutical composition of the invention, the less soluble penetrant component is a lipid, preferably a polar lipid and the more soluble component is a surfactant or a surfactant-like molecule or else such form of polar lipid which is sufficiently soluble for the purpose of this invention.

In another preferred embodiment of the use or of the pharmaceutical composition of the present invention, the average penetrant diameter is between 25 nm and 500 nm, preferably between 30 nm and 250 nm, even more preferably between 35 nm and 200 nm and particularly preferably between 40 nm and 150 nm.

In a different preferred embodiment of the use or of the pharmaceutical composition of the present invention the penetrant concentration in the formulation for the use in the human or animal nose is 0.001 weight-% (w-%) to 20 w-% of total dry mass in the formulation, in particular between 0.01 w-% and 15 w-%, more preferably between 0.1 w-% and 12.5 w-% and most preferred between 0.5 w-% and 10 w-%.

In a further preferred embodiment of the use or of the pharmaceutical composition of the present invention the supporting medium, e.g. a buffer, is selected to be a biocompatible solution with an osmotic activity similar to that of a monovalent electrolyte with a concentration range between 1 mM and 500 mM, more preferably between 10 mM and 400 mM, even more preferably between 50 mM and 300 mM, and most preferably between 100 mM and 200 mM or else such solution that affords practically sufficient penetrant stability combined with practically sufficient transport rate across the barrier. The term "practically sufficient penetrant stability" means that the penetrant stability meets the reasonable product quality criteria. The term "practically sufficient transport rate" means that enough drug is transported through the barrier without using unreasonably large application volume or time. Said sufficient penetrant stability combined with sufficient transport rate across the barrier can be determined by the person skilled in the art without undue experimentation.

In another preferred embodiment of the use or of the pharmaceutical composition of the present invention, the relative drug or agent concentration is between 0.001 w-% and 40 w-% of total penetrant mass, in particular between 0.01 w-% and 30 w-%, even better between 0.1 w-% and 25 w-% and most preferably between 0.5 w-% and 15 w-%.

In one further preferred embodiment of the use or of the pharmaceutical composition of the present invention the medium supporting the drugs and carriers is a biocompatible buffer with pH value between 4 and 10, more frequently between 5 and 9 and most often between 6 and 8.

In another preferred embodiment of the use or of the pharmaceutical composition of the present invention additives are included in said composition to reduce the system sensitivity to chemical, biological or ambient stress, including anti-oxidants, antagonists of undesired enzyme action, cryo-preservants, microbicides, etc., or else modulators of physically important system properties, such as formulation viscosity, etc.

In a different preferred embodiment of the use or of the pharmaceutical composition of the present invention the relative drug or agent dose to be administered non-invasively through the nose by means of highly adaptable carriers is chosen to be between 0.1x and 500x, more often between 0.5x and 250x, and even more preferably between 1x and 100x different from the corresponding drug or agent dose that would have to be injected to achieve the desired biological effects. Again, the latter dose can be determined by the person skilled in the art without undue experimentation and on the basis of his common general knowledge.

In another preferred embodiment of the use or of the pharmaceutical composition of the present invention the applied penetrant dose is between 0.01 mg and 15 mg per nostril, even more often is in the range 0.1 mg and 10 mg per nostril, and preferably is between 0.5 mg and 5 mg per nostril.

The efficiency of administration and the biological effects of the agent or drug chosen, consequently, can be controlled by using different application volumes. Various metered delivery devices can be used for the purpose.

Accordingly, in an additional preferred embodiment of the use or of the pharmaceutical composition of the present invention said formulation is administered using a metered delivery device.



In one further preferred embodiment of the use or of the pharmaceutical composition of the present invention different application volumes are selected to control the efficiency of administration and the biological effects of the chosen agent or drug.

In a different preferred embodiment of the use or of the pharmaceutical composition of the present invention the penetrants in suspension are loaded with the drugs or agents within 24 hours prior to the formulation administration, preferably 360 min, more preferably 60 min and even more preferably 30 min before the resulting formulation is administered in the nose. This embodiment is expected to improve the formulation stability, loading efficiency, the release kinetics, ease of use, compliance, etc.

In another preferred embodiment of the use or of the pharmaceutical composition of the present invention the, delivery device is loaded at the treatment site.

In a further preferred embodiment of the use or of the pharmaceutical composition of the present invention the delivery device is loaded separately with penetrants and the molecules, particularly biological agents, to be associated therewith.

In one further preferred embodiment of the use of the present invention wherein the pharmaceutically active ingredient is for administration to the nervous system.

The term "administration" in connection with this embodiment means that the pharmaceutical composition is transnasally applied, but the target site of the active ingredient is the nervous system, preferably the CNS and most preferably the brain. The possibility to use nasal application of the highly adaptable, drug-loaded penetrants in the nose to mediate a practically useful transfer of the drug across the barrier can thus be exploited to transport a meaningful amount of the drug, and to create a significant concentration of such drug, in the central nervous system or some other adjacent tissue, such as the eye.

In another preferred embodiment of the invention, the pharmaceutical composition of the invention is a vaccine.

Said vaccine can be used for therapeutic or prophylactic vaccination.

The term „(therapeutic) vaccination“ in the context of this invention describes any kind of therapeutic immunisation, whether done after the disease has been already established, to improve a clinical situation, or else for the purpose of preventing a disease. Such a vaccination can involve single or repeated administration(s) of the vaccine of the invention. Therapeutic vaccination will either prevent a pathological situation and/or improve a clinical situation. When applied as a preventive agent, it will generally result in a protective immune response.

Immunisation denotes any kind of provoking an immune response, irrespective of whether said response is therapeutic or non-therapeutic.

An „antibody“ or an „immunoglobulin“ denotes an IgA, IgD, IgE, IgG, or IgM, including all subtypes, such as IgA1 and IgA2, IgG1, IgG2, IgG3, IgG4. Their “derivatives” include chemical, biochemical and otherwise obtainable derivatives, such as genetically engineered antibody derivatives. Fragments include e.g. single chain fragments, Fc-, Fab- F(ab')<sub>2</sub>- and other parts of Ig-s, independent of whether they are of endogenous, xenogenic, (semi)synthetic or recombinant origin. Also comprised by the invention are complexes of two or more of the above-recited antibodies, derivatives or fragments.

The term „immunogen“ denotes a hapten coupled to an immunological carrier or an antigen, free or associated with a carrier, which is capable of inducing an immune response.

„Immuno-tolerance“ denotes the lack or, more generally, the reduction of an undesired immune response to an antigen.

Th1 (T-helper cell type I) related antibodies include IgG2a, IgG2b and IgG3.

Th2 (T-helper cell type II) related antibodies comprise the classes of IgG1, IgG4 and IgE.

As has been indicated above, the successful immunisation with the vaccine of the invention through the nose is a significant step forward in the design of conveniently administrable vaccines that (a) are highly efficient over a wide range of immunogens of

varying size and properties; (b) can be formulated together with certain cytokines, compounds that mediate cytokine activity or compounds that antagonize cytokine activity in order to specifically direct the corresponding immune response or to augment or suppress the same as may be desired; (c) do not depend on the perturbing injection by a needle; and (d) cause no irritating side effects. In addition, with the vaccine of the invention, successful tolerogenesis may be achieved.

It has inter alia been found in accordance with the present invention that

- Tween-SPC micelles give protection significantly below that of the vaccine of the present invention, suggesting that the small size of the carrier or the presence of surfactants alone does not suffice for a successful immunisation;
- orally administered immuno-carriers create lower specific antibody titers than the transnasally administered vaccine of the invention, as determined on the basis of absorbance measurements;
- the transnasal vaccine of the invention gives rise to higher specific IgG1 and IgG2 titers in the blood and to comparable IgG2a and IgM titers as compared to mixed micelles; all titers were, on top of this, higher than those generated by immunisation with SPC:cholesterol (1:1) liposomes.

When the transnasal vaccine of the invention is formulated together with a cytokine or an immunoadjuvant it is advantageous to use (blends of) bacterial extracts. Specific examples given in this application include monophosphoryl lipid A (MPL) and IL-12 or GM-CSF and IL-4. In principle, however, the vaccine of the invention may be formulated or applied together with any of the compounds mediating, inducing or displaying cytokine activity or with antagonists thereto that have been recited herein above.

It is preferred that the vaccine of the invention further comprises a pathogen extract or a compound from a pathogen or a fragment or a derivative thereof.

Most preferably, said pathogen extract or compound is selected from hepatitis virus, (human) immunodeficiency virus, herpes viruses, small-pox (chicken-pox), influenza, measles, mumps or polio viruses, cytomegalovirus, rhinovirus, etc., or fungi prospering inside host cells, a parasite including animal parasites, such as protozoa and helminths, and ectoparasites, such as ticks and mites, or *Brucella* species, including





In a different preferred embodiment of the vaccine of the present invention the relative immunogen/antigen dose to be administered non-invasively through the nose by means of highly adaptable carriers is chosen to be between 0.01x and 100x, more often between 0.05x and 75x, and even more preferably between 0.1x and 50x different from the corresponding immunogen/antigen that would have to be injected to achieve the desired biological effect. Again, the latter dose can be determined by the person skilled in the art without undue experimentation and on the basis of his common general knowledge.

It is further preferred in accordance with the invention that in said vaccine the concentration of the transnasally administered adjuvant is between 10x lower and up to 1000x higher than used with the corresponding subcutaneously injected formulations employing similar antigen, the transnasally administered immunoadjuvant concentration more often differing from the injected immunoadjuvant concentration by the factor between 0.5 and 100, or better, by the factor between 1 and 50, and best between 2 and 25.

The invention also relates to a container comprising the pharmaceutical composition recited herein-above. The unit dosage may be determined according to the desired application.

Furthermore, the invention additionally relates to a package comprising at least one container comprising the pharmaceutical composition as described above. The package of the invention can comprise one, two, three, four or more vials/units of the pharmaceutical composition of the invention.

The invention finally relates to methods of treating a patient in need thereof comprising transnasally administering any of the above recited pharmaceutical compositions.

The present invention further relates to a method for generating a protective or tolerogenic immune response on a mammal by vaccinating said mammal with a vaccine as described above.

In a preferred embodiment of the method according to the present invention different administration volumes are selected to control the applied immunogen dose and the



In another preferred embodiment of the method according to the present invention the flux of penetrants that carry an immunogen through the various pores in a well-defined barrier is determined as a function of the suitable driving force or a pressure acting across the barrier and the data are then conveniently described by a characteristic curve which, in turn, is employed to optimise the formulation or application further.

The disclosure content of the documents cited throughout this specification are herewith incorporated by reference. Further incorporated by reference is the complete disclosure content of the co-pending application filed in the name of IDEA AG and bearing the title "Noninvasive vaccination through the skin".

The figures show:

Figure 1 illustrates the effect of nasal insulin administration by means of carriers in an insulin dependent diabetes mellitus patient, with the result of an i.v. injection of fast-acting insulin (Actrapid, Novo-Nordisk) shown in the inset for reference.

Figure 2 illustrates the glucodynamics in a healthy human volunteer following intranasal administration of insulin by means of Transfersomes. Inset gives the result of intravenous injection of similar formulation for reference purpose.

Figures 3a and 3b provide further examples measured with a healthy volunteer following intranasal administration of insulin formulations with inferior characteristics, believed to be due to too slow drug release from the carrier.

Figure 4 illustrates the capability of nasally administered cytokines, associated with Transfersomes, to affect the outcome of transnasal immunisation with tetanus toxoid.

Figure 5 illustrates the biodistribution of insulin-derived radioactivity in mice following nasal administration of the agent in transfersomes.

Figure 6 gives the corresponding results for interferon, as measured in mice.



Figures 7 illustrate the effect of changing aggregate size and/or deformability on TT specific immune response in mice treated with various mixed micelles, Transfersomes or liposomes loaded with TT. Panels a and b show antibody isotype patterns, and in panel c the total antibody titre, as expressed in absorbency change is given.

Figures 8 highlight the (small) effect of changing antigen dose (in the high dosage range) on transnasal immunisation of mice with TT by means of Transfersomes with or without lipid A derivative as an immunoadjuvant. In panel a, the results of total absorbance measurements are given, panel b shows the corresponding titration curves, and panel c gives the relevant antibody isotypes.

Figure 9 is organised in similar fashion to compare the outcome of intranasal, oral or subcutaneous TT administration using different antigen doses and purity.

Figure 10: For comparison, animal protection (survival) data are given for the experiments in which several doses and administration routes were compared.

Figure 11 presents a set of data on the effect of various cytokines, or their combination, on the murine immune response to TT administered into the nose by means of transfersomes, with s.c. data given for comparison. Panel a gives the absorbance and titre data and panel b contains the isotype distribution results.

Figure 12 deals with the effects of combining low and high molecular weight immuno-adjuvants (lipid a analogue and interleukin-12).

Figure 13 illustrates the effect of specific cytokine inducers of microbial origin. Cholera toxin (CT) is used for the purpose.

Figure 14 shows one effect of heat labile toxin from *E. coli* as immuno-adjuvant.

Figure 15 illustrates the results obtained with a combination of two antigens, tetanus toxoid and cholera toxoid.









apparent bioavailability of nasal insulin based on these data is around 4% and, consequently, somewhat lower than that reported in example 1. This may have to do with the presumed variability in drug release between different formulations which is illustrated in the following examples.

Nasal administration of carrier-associated insulin, according to the test person, caused no adverse side effect, locally or systemically.

**Examples 3-5:**

*Insulin associated with suboptimal carriers*

*Carriers*

as in previous examples, but believed not to release the drug readily owing to the higher affinity of selected insulin batch for the carrier, which makes the drug adsorption irreversible.

Applied doses: 50 IU, 50 IU

**Results** of the test measurements done with several different vesicle suspensions, illustrated in figure 3, signal lack of action for the insulin administered nasally with such carriers. The blood glucose concentration in the investigated normoglycaemic test person remains the same before, during and after the drug administration, for several hours at least. This suggests that the mere presence of carriers, or their ingredients, is insufficient to improve the bioavailability of nasally applied macromolecules, such as insulin. To achieve the desired biological effect, the rate of drug release from the carrier must also be adequate, such rate being determined in dedicated ex vivo experiments by using conventional protein binding deassociation techniques.

## Animal experiments

### Examples 6-9 :

#### *Labelled insulin delivery across nasal mucosa of test mice*

highly adaptable penetrants:

87.4 mg/mL phosphatidylcholine from soy bean (SPC)

12.6 mg/mL of a 50 % ionised cholic acid

phosphate buffer, 50 mM, pH 6.5

hr-Insulin (Actrapid™, Novo-Nordisk)

labelled insulin from Amersham

(345  $\mu$ L contain 1.08  $\mu$ g insulin and 1.725 mg BSA)

<sup>125</sup>I-labelled insulin (210  $\mu$ L) was mixed with 210  $\mu$ L of hr-insulin (Actrapid™ Novo-Nordisk, 100 HM) and purified 2 times by centrifugation to eliminate the non-bound label, which diffuses across the barrier much faster and better than whole drug molecules. 100  $\mu$ L of the resulting solution was mixed with 150  $\mu$ L phosphate buffer to yield pH around 7. Protein solution and lipids were processed together, bringing the final vesicle size by repeated extrusion through 100 nm pore filters to values around 150 nm.

Mice of NMRI strain (36 g to 51 g) from a local supplier were kept in suspension cages in groups of 4 to 6. The animals had free access to standard chow and water. Each mouse received 2.5  $\mu$ L of labelled penetrant suspension containing insulin per nostril. Then, the decrease in total radioactivity was assessed by whole-body camera at least 2 times. At different times the mice were killed and all major organs were taken and measured separately. The carcass was measured in two steps, after organ elimination and then after separation of the head. Radioactivity in excrement and cage was also determined.

**Results** pertaining to different time-points are given in Figure 4. They show that substantial amount of nasally administered radioactivity is recovered from the body, even after exclusion of gastro-intestinal tract, especially during the first hours following

suspension administration. Values in the blood are in the range of 9% at 0.5 h and 2%, the specific concentration falling from 3%/mL at the beginning to 0.7%/mL at the end. Activity in the nose decreases from 10.4% at 0.5 h to 0.3% at 8 h. Liver values are between 2.3% after 0.5 h, the maximum around 2.8 at 1 h and values above 1% after 4 h. After 8 h, the residuum in the liver is around 0.4%. The relatively high hepatic values are suggestive of passage of particles, that is, penetrants, through the barrier and subsequent uptake in the reticulo-endothelial system.

Corresponding CNS values are 0.1% and 0.03%. Maximum in the brain is measured between the first and second hour with app. 0.11% and 0.14%, respectively, which amounts to around 0.3%/g organ. These, apparently low values compare favourably with the result of more conventional drug delivery into CNS which yields values below 0.5% of injected dose or around 0.15%/g organ, for example, when transferrin-receptor is used to deliver the drug (Pasechnik & Price, 1996). In the case of white-germ agglutinin 0.1% was found in olfactory bulb.

### Examples 10-11 :

highly adaptable penetrants

87.4 mg/mL phosphatidylcholine from soy bean (SPC)

12.6 mg/mL of a 50% ionised cholic acid

phosphate buffer, 50 mM, pH 6.5

human recombinant insulin (Actrapid™, Novo-Nordisk)

labelled insulin from Amersham

In a related experiment, 345  $\mu$ L of <sup>125</sup>I-labelled insulin was mixed with 345  $\mu$ L of cold Actrapid™ (Novo-Nordisk) and purified 2 times, as in previous experiment. After addition of 200  $\mu$ L phosphate buffer, 150  $\mu$ L of resulting solution was mixed with the lipids and extruded to final vesicle size. The applied dose was 3  $\mu$ L per nostril. Mice were killed after 1 h, fixed, cut in thin sections and inspected by the whole-body radiography. Free insulin in solution was used for comparison.

The results of above mentioned experiments (not shown) revealed high label accumulation in the nasal region, as one would expect, substantial spill-over into the GI





highly adaptable penetrants

37.7 mg/mL phosphatidylcholine from soy bean (SPC)

62.3 mg/mL polysorbate (Tween 80)

phosphate buffer, 10 mM, pH 6.5

Tetanus toxoid, as antigen (2 mg/mL)

Interferon- $\gamma$  (IFG- $\gamma$ )

Granulocyte-monocyte-colony stimulating factor (GM-CSF)

Interleukin 4 (IL-4)

Interleukin 12 (IL-12)

Applied dose: 3  $\mu$ L per nostril

Mice of Swiss albino strain (18-20 g) were obtained from The National Institute of Nutrition (Hyderabad, India). They were 8 to 12 weeks old at the time of first immunisation. The antigen alone or in combination with various cytokines, both believed to be at least partly associated with the carriers, was positioned with a sequencing in front of the animal nose and left to be sucked-in by the latter. Blood samples were collected retro-orbitally and tested with specific antibodies directed against the employed antigen by measuring absorbance at 492 nm, after subtraction of blank samples with ELISA.

The results of above mentioned measurements, illustrated in figure 6, suggest that the presence of all tested cytokines in vaccination formulation, based on the highly adaptable antigen carriers, increases the serum absorbance compared to that characterising the non-modulated value, determined after simple immuno-carrier administration. Relative differences are more likely consequences of diverse bio-potency of tested immuno-modulants employed in the present specific experimental system than indicative of variable macromolecular transport rate across the nasal mucosa.

The observed 100% increase in serum absorbance measured for GM-CSF/IL-4 combination is remarkable, as it is known that neither polysorbate nor phosphatidylcholine ex soy-bean can markedly enhance permeation capability on their own. It is therefore reasonable to assume that the observed effect is not simply due to the delivery of antigen molecules (with the molar mass of 150 kDa) across the nasal

mucosa but, moreover, testify that at least a proportion of co-administered cytokines has passed the barrier in a biologically active form.

### **Examples 20-21:**

highly adaptable penetrants

as in examples 14-19, except for the absence of cytokines

Tetanus toxoid antigen (2 mg/mL)

Mixed lipid micelles

14.8 mg/mL phosphatidylcholine from soy bean (SPC)

85.2 mg/mL polysorbate (Tween 80)

phosphate buffer, 10 mM, pH 6.5

Tetanus toxoid antigen (2 mg/mL)

Applied dose: 3  $\mu$ L per nostril

Experiments were done as described with previous examples (14-19).

Immune response in the animals treated with mixed lipid micelles as in Examples 14-19 was clearly inferior to that measured after the nasal application of antigen in the highly adaptable lipid vesicles, despite the fact that the latter contained a smaller amount of Tween 80 than the former. If the surfactant was responsible for the transport of macromolecules across nasal mucosa, owing to its action as skin permeation enhancer, precisely the opposite experimental outcome would have been expected.

This suggests that highly adaptable carriers (mixed lipid vesicles) transport macromolecules across the nasal mucosa by a mechanism other than the drug permeation.



65 mg phosphatidylcholine from soy bean (SPC)  
35 mg cholesterol  
0.9 mL phosphate buffer, pH 6.5

Tetanus toxoid (2 mg/mL; home made) used at the dose of  
40  $\mu$ g (20  $\mu$ L) TT per mouse and immunisation

The medium filtrate from a culture of *Clostridium tetani* grown in vitro was used as an purified antigen. Pure toxoid was purchased from Accurate Antibodies, NY, USA.

To test the effect of aggregate properties in the formulation, three kind of aggregates were prepared: relatively large vesicles (diameter between 100 nm and 200 nm) either comprising a flexible membrane (Transfersomes) or a relatively rigid membrane (liposomes) and much smaller micelles (diameter below 50 nm). The latter were chosen to mimick the more conventional approach of using detergents as nasal mucosa permeation enhancers.

Amongst the eight tested formulations, Transfersomes, on the average, give best results, but absolute titres are always very low, probably owing to the antigen impurity. Mixed lipid micelles are most efficient in creating IgA, but are not really different than the other aggregates in the case of IgG2a and IgM, whilst in the case of Ig2b they are comparable to Transfersomes. The IgG1 level, which is decisive for animal protection, is only significantly elevated when Transfersomes are used to deliver TT across through the nose (see figure 7a).

Mixed micelles containing less potent detergents (with lesser skin permeation enhancing capability) are, relatively speaking, less efficient 'immuno-carriers' (see figure 7b), the more deformable Transfersomes with a higher Tw content standing clearly out in the case of IgG2a and IgM, are similar to the less deformable Transfersomes with a lower Tw content in the case IgG1 and IgG3, and are as efficient as mixed micelles with Tw in the case of IgA and IgG2b. The smallness of measured values is reason for the concern, however, which can best be overcome by using purer antigen.

Looking at the cumulative titre of all specific anti-TT antibodies in the serum, liposomes are relatively efficient 'immuno-carriers' in the primary and mature response (perhaps owing to the action of non-associated TT), whilst the Tw rich mixed micelles are the worst. NaCh Transfersomes are top performers in the late immune response (cf. figure 7c).

### **Examples 30-35:**

#### Antigen dose and purity effect

Highly deformable vesicles (Transfersomes):

86.3 mg phosphatidylcholine from soy bean (SPC)

13.7 mg sodium cholate (NaChol)

+/- 0.04 mol-% monophosphoryl Lipid A (LA) relative to SPC

0.9 mL phosphate buffer, 10 mM, pH 6.5

Tetanus toxoid (TT, from local source, purified by ultrafiltration)

0  $\mu$ g, 40  $\mu$ g or 80  $\mu$ g TT/ mouse/ immunisation

To obtain partially purified antigen, such filtrate was passed through a 10 kDa cut-off membrane and washed thoroughly with phosphate buffer, pH 6.5; in the process, the culture filtrate was concentrated 15 times.

Dose dependence results are illustrated in figure 8a. The TT-specific increase in serum absorbance following TT administration through the nose by means of Transfersomes reveals a positive dose dependence in the primary and late immune response in the absence of LA, the presence of LA reverting this trend. Titre-wise and with regard to specific antibody isotype distribution, similar but not identical picture is obtained (cf. figures 8b and 8c). The survival data are indicative of good protection in every case. Taken together this suggests that the required dose for non-invasive nasal immunisation by means of highly deformable carriers is much lower than that required for a successful non-invasive TT administration through the skin.









86.3 mg phosphatidylcholine from soy bean (SPC)

13.7 mg sodium cholate (NaChol)

0.9 mL phosphate buffer, 10 mM, pH 6.5

0.1 mL ethanol

TfsT

36 mg phosphatidylcholine from soy bean (SPC)

64 mg Tween 80

0.9 mL phosphate buffer, 10 mM, pH 7

Cholera toxin (CT; Sigma, Neu-Ulm), 2  $\mu$ g/immunisation, if specified,

Tetanus toxoid (TT, pure; Accurate Antibodies), 2 mg/mL.

Volume doses corresponding to 0  $\mu$ g TT/mouse/immunisation (negative control), 1  $\mu$ g TT/mouse, 5  $\mu$ g TT/ mouse, 10  $\mu$ g TT/ mouse, 20  $\mu$ g TT/mouse, 40  $\mu$ g TT/mouse (in the absence of CT) and 0.5  $\mu$ g TT/mouse/immunisation, 1  $\mu$ g TT/mouse, 5  $\mu$ g TT/ mouse (when using CT) was used intranasally in the type T Transfersomes (TfsT) in both nostrils and at the dose of 0.5  $\mu$ g TT/mouse/immunisation in the type C Transfersomes (TfsC) in 4-6 Swiss albino mice. Moreover, 20  $\mu$ g TT/ mouse/immunisation in TfsT was injected subcutaneously at the corresponding site in the positive control group. Immunisations were done on days 1, 14, 28.

The protective effect of antigen applied in the nose was good when the antigen dose exceeded 20  $\mu$ g/immunisation; lower doses yielded insufficient, but detectable protection (cf. figure 13). When cholera toxin (CT) was included into the test formulation together with the tetanus toxoid, excellent protection was achieved already at the lowest of tested doses (0.5  $\mu$ g/immunisation), independent of the ultra-deformable carrier composition. Protection was complete in all test groups containing CT in the formulation applied on the skin.

**Examples 72-74:**

Heat labile toxin from E. coli (HLT) as immuno-adjuvant

Highly deformable vesicles, Transfersomes™:

TfsC

86.3 mg phosphatidylcholine from soy bean (SPC)

13.7 mg sodium cholate (NaChol)

0.9 mL phosphate buffer, 10 mM, pH 6.5

Heat Labile Toxin (HLT, SIGMA, Neu-Ulm),  $\leq 1$  mg/mL plus, if required

Tetanus Toxoid (TT, pure, Accurate Antibodies) 2 mg/mL

or

TfsT

36 mg phosphatidylcholine from soy bean (SPC)

64 mg Tween 80

0.9 mL phosphate buffer, 10 mM, pH7

Heat Labile Toxin (LT, SIGMA, Neu-Ulm),  $\leq 1$  mg/mL, if required,

Tetanus Toxoid (TT, pure, Accurate Antibodies) 2 mg/mL

Antigen carriers were prepared and all experiments (with Swiss albino mice) and assays were carried out as described in the previous examples. A series of different HLT doses ranging from approx. 50 ng/application to multi-microgram quantity per application was used in combination with TT concentrations in the range of approx. 100 ng and up to 10  $\mu$ g per application. In most cases, volume doses corresponding to 0.5  $\mu$ g TT / mouse / i.n. immunisation and 0.1 to 0.5  $\mu$ g HLT / mouse / i.n. immunisation and a positive control with 0.5  $\mu$ g TT for s.c. injection was used for mice immunisation.

As is shown in Figure 14, anti-TT titres are improved by HLT acting as an adjuvant in comparison with the result of s.c. injection without an adjuvant. The humoral response is dose dependent, i.e. higher anti-TT titres are achieved with the higher dose of HLT. The correlation is not linear, however, but shows rather a maximum (data not shown).

Protection against tetanus toxin challenge is equally efficient for the high and the low dose of immuno adjuvant.

This suggests that adjuvants should be used in the nose in conjunction with Transfersomes at maximum at the dose that is in the low-end range of doses used for conventional invasive (s.c.) immunisations; the minimum dose for immuno-Transfersomes usage in the nose should also be 1-2 orders lower, for the adjuvant tested in this series for most, if not all, immunoadjuvants.

**Example 75:**

Bivalent vaccination with Tetanus Toxoid and Cholera Toxin as antigens

Highly deformable vesicles, Transfersomes™:

86.3 mg phosphatidylcholine from soy bean (SPC)

13.7 mg sodium cholate (NaChol)

0.9 mL phosphate buffer, 10 mM, pH 6.5

Cholera Toxin (CT, SIGMA, Neu-Ulm), ≤ 1 mg/mL

Tetanus Toxoid (TT, pure, accurate antibodies) 2 mg/mL

or

TfsT

36 mg phosphatidylcholine from soy bean (SPC)

64 mg Tween 80

0.9 mL phosphate buffer, 10 mM, pH7

Cholera Toxin (CT, SIGMA, Neu-Ulm), ≤ 1 mg/mL

Tetanus Toxoid (TT, pure, accurate antibodies) 2 mg/mL

Details on the formulation and its preparation, on vesicle characterisation and on animal experiments as well as the following assays are given in the description of related examples hereinabove.

The main result of this experimental series was that Cholera Toxin, added to the formulation as an adjuvant, can also induce the formation of anti-CT antibodies in practically relevant quantity. This effect can, but does not have to, be achieved using concentrations of CT and TT sufficient to ensure good protection against the challenge with tetanus toxin. Figure 15 compares anti-TT and anti-CT titres from the same mice immunised with TT and CT within the same carrier. This reveals the potential of Transfersomes™ containing more than one antigen to serve as the basis for at least bivalent vaccines.



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CLAIMSREPLACED BY  
ART 34 AMDI

1. Use of a penetrant, suspended or dispersed in a solvent, in the form of a minute fluid droplet surrounded by a membrane-like coating of one or several layers of at least two different substances or two different forms of a substance with the tendency to aggregate, said substances or forms of a substance differing by at least the factor of 10 in the solubility in a preferably aqueous, liquid medium, such that the average diameter of homo-aggregates of the more soluble substance or form of the substance or the average diameter of the hetero-aggregates consisting of both said substances or forms of said substance is smaller than the average diameter of homo-aggregates of the less soluble substance or form of the substance and/or wherein the more soluble component tends to solubilise the penetrating droplet and wherein the content of such component amounts to up to 99 mol-% of the concentration required to solubilise the droplet or else corresponds to up to 99 mol-% of the saturating concentration in the un-solubilised droplet, whichever is higher, and/or wherein the elastic deformation energy of the droplet surrounding the membrane-like coating is at least 5x lower, more preferably is at least 10x lower and ideally is more than 10x lower than that of the red blood cells or of the phospholipid bilayers with fluid aliphatic chains, such droplets then acting as carriers for the transnasal administration of a pharmaceutically active compound, an antigen, an allergen, a mixture of antigens and/or a mixture of allergens.
  
2. Use of a penetrant, suspended or dispersed in a solvent, in the form of a minute fluid droplet surrounded by a membrane-like coating of one or several layers of at least two different substances or two different forms of a substance with the tendency to aggregate, said substances or forms of a substance differing by at least the factor of 10 in the solubility in a preferably aqueous, liquid medium, such that the average diameter of homo-aggregates of the more soluble substance or form of the substance or the average diameter of the hetero-aggregates consisting of both said substances or forms of said substance is smaller than the average diameter of homo-aggregates of the less soluble substance or forms of the substance and/or wherein the more soluble component tends to solubilise the penetrating droplet and wherein the content of such component amounts to up to 99 mol-% of the concentration required to

solubilise the droplet or else corresponds to up to 99 mol-% of the saturating concentration in the un-solubilised droplet, whichever is higher, and/or wherein the elastic deformation energy of the droplet surrounding the membrane-like coating is at least 5x lower, more preferably is at least 10x lower and ideally is more than 10x lower than that of the red blood cells or of the phospholipid bilayers with fluid aliphatic chains as a carrier for the preparation of a pharmaceutical, preferably a vaccine composition for transnasal administration.

3. Use of a penetrant, suspended or dispersed in a solvent, in the form of a minute fluid droplet surrounded by a membrane-like coating of one or several layers of at least two different substances or two different forms of a substance with the tendency to aggregate, said substances or forms of a substance differing by at least the factor of 10 in the solubility in a preferably aqueous, liquid medium, such that the average diameter of homo-aggregates of the more soluble substance or form of the substance or the average diameter of the hetero-aggregates consisting of both said substances or forms of said substance is smaller than the average diameter of homo-aggregates of the less soluble substance or form of the substance and/or wherein the more soluble component tends to solubilise the penetrating droplet and wherein the content of such component amounts to up to 99 mol-% of the concentration required to solubilise the droplet or else corresponds to up to 99 mol-% of the saturating concentration in the un-solubilised droplet, whichever is higher, and/or wherein the elastic deformation energy of the droplet surrounding the membrane-like coating is at least 5x lower, more preferably is at least 10x lower and ideally is more than 10x lower than that of the red blood cells or of the phospholipid bilayers with fluid aliphatic chains, said penetrant being used in combination with a pharmaceutically active ingredient or an allergen or an antigen for the preparation of a transnasally administerable pharmaceutical composition for the treatment of infective diseases, endocrine disorders, preferably hypopituitarism, diabetes, hyperthyroidism, thyroiditis, most preferably Hashimoto's thyroiditis, subacute thyroiditis; adrenal disorders, preferably Addison's disease, secondary adrenal insufficiency, Cushing's syndrome; gastrointestinal disorders, preferably Crohn's disease, colitis; hemorrhagic diseases, preferably hemophilia, leukopenia, hypereosinophilic syndrome; musculoskeletal and connective tissue disorders, preferably rheumatoid arthritis, Sjögren's syndrome, Bechet's

syndrome, lupus, scleroderma, polymyositis/dermatomyositis, polymyalgia rheumatica and temporal arthritis, polyarteriosis nodosa, Wegener's granulomatosis, mixed connective tissue disorder, ankylosing spondylitis, psoriatic arthritis, osteoarthritis, Paget's disease, sciatica, bursitis, tendonitis and tenosynovitis, epicondylitis, fibromyalgia, eosinophilic fasciitis; neurological disorders, preferably pain, singultus, vertigo, seizure disorders, sleep disorders, transient ischemic attacks, spinal cord injury, demyelinating diseases, nerve root disorders, myasthenia gravis; oncological disorders; psychiatric disorders, preferably drug dependence, neuroses, mood disorders, schizophrenic disorders, delusional disorders; and/or for use in the field of gynecology, preferably for the treatment of dysmenorrhea, menopause, chronic anovulation, premature ovarian failure, endometriosis, infertility; and/or for use in the field of immunology, preferably transplant rejection, hyposensitisation, allergen immunotherapy or prophylactic vaccination.

4. A pharmaceutical composition for transnasal administration comprising a carrier, which is a penetrant, suspended or dispersed in an aqueous solvent, in the form of a minute fluid droplet surrounded by a membrane-like coating of one or several layers of at least two different substances or two different forms of a substance with the tendency to aggregate, said substances or forms of a substance differing by at least the factor of 10 in solubility in a preferably aqueous, liquid medium, such that the average diameter of homo-aggregates of the more soluble substance or form of the substance or the average diameter of the hetero-aggregates consisting of both said substances or forms of said substance is smaller than the average diameter of homo-aggregates of the less soluble substance or form of the substance and/or wherein the more soluble component tends to solubilise the penetrating droplet and wherein the content of such component amounts to up to 99 mol-% of the concentration required to solubilise the droplet or else corresponds to up to 99 mol-% of the saturating concentration in the un-solubilised droplet, whichever is higher, and/or wherein the elastic deformation energy of the droplet surrounding the membrane-like coating is at least 5x lower, more preferably is at least 10x lower and ideally is more than 10x lower than that of the red blood cells or of the phospholipid bilayers with fluid aliphatic chains said composition also including a

pharmaceutically active ingredient, an allergen, an antigen, a mixture of antigens and/or a mixture of allergens.

5. The use of claim 3 or the pharmaceutical composition of claim 4 wherein the pharmaceutically active ingredient is an adrenocorticostaticum, an adrenolyticum, an androgen or antiandrogen, an antiparasiticum, an anabolicum, an anaestheticum or analgesicum, an analepticum, an anti-allergicum, antiarrhythmicum, antiarteroscleroticum, antiasthmaticum and/or bronchospasmolyticum, an antibioticum, an anti-infective agent, antidepressivum and/or antipsychoticum, an antidiabeticum, an antidot, an antiemeticum, antiepilepticum, antifibrinolyticum, anticonvulsivum or anticholinergicum, an enzyme, a coenzyme or the corresponding enzyme inhibitor, an antihistaminicum (and combinations thereof) or antihypertonicum, an antihypotonicum, anticoagulant, antimycoticum, antimyasthenicum, an agent against Morbus Alzheimer or Morbus Parkinson, an agent for ACS therapy, an antiphlogisticum, antipyreticum, antirheumaticum, antisepticum, a respiratory analepticum or a respiratory stimulant, a broncholyticum, cardi tonicum, chemotherapeuticum, a coronary dilatator, a cytostaticum, a diureticum, a ganglium-blocker, a glucocorticoid, an anti-flew agent, a haemostaticum, hypnoticum, an immunoglobuline or its fragment or any other immunologically active substance, such as an immunomodulator, a bioactive carbohydrate (derivative), a contraceptive, an anti-migraine agent, a corticosteroid, a muscle relaxant, a narcoticum, a neurotherapeuticum, a (poly)nucleotide, a neurolepticum, a neurotransmitter, a (poly)peptide (derivative), an opiate, an ophthalmicum, (para)-sympaticomimeticum or (para)sympathicoliticum, a protein(derivative), a psoriasis/neurodermitis drug, a mydriaticum, a psychostimulant, rhinologicum, a sleep-inducing agent, a sedating agent, a spasmolyticum, tuberculostaticum, urologicum, a vasoconstrictor or vasodilatator, a virustaticum, a wound-healing substance, an alcohol abuse preparation, an anticonvulsant, an antineoplastic, an antirheumatic, an appetite suppressant, a biological response modifier, a blood modifier, a bone metabolism regulator, a cardioprotective agent, a cardiovascular agent, a central nervous system stimulant, an enzyme, an agent for erectile dysfunction therapy, a fertility agent, a gastrointestinal agent, a gout preparation, a hormone, an agent for hypercalcemia management, an agent for hypocalcemia management,











mM or else such solution that affords practically sufficient penetrant stability combined with practically sufficient transport rate across the barrier.

23. The use of any one of claims 1 to 22 or the pharmaceutical composition of any one of claims 4 to 22 wherein the relative drug or agent concentration is between 0.001 and 40 weight-% of total penetrant mass, in particular between 0.01 w-% and 30 w-%, even better between 0.1 w-% and 25 w-% and most preferably between 0.5 w-% and 15 w-%.
24. The use of any one of claims 1 to 23 or the pharmaceutical composition of any one of claims 4 to 23 wherein the medium supporting the drugs and carriers is a biocompatible buffer with pH value between 4 and 10, more frequently between 5 and 9 and most often between 6 and 8.
25. The use of any one of claims 1 to 24 or the pharmaceutical composition of any one of claims 4 to 24 wherein the additives are included in the preparation to reduce the system sensitivity to chemical, biological or ambient stress, including anti-oxidants, antagonists of undesired enzyme action, cryo-preservants, microbicides, etc., or else modulators of physically important system properties, such as formulation viscosity, etc..
26. The use of any one of claims 1 to 25 or the pharmaceutical composition of any one of claims 4 to 25 wherein the relative drug or agent dose to be administered non-invasively through the nose by means of highly adaptable carriers is chosen to be between 0.1x and 500x, more often between 0.5x and 250x, and even more preferably between 1x and 100x different from the corresponding drug or agent dose that would have to be injected to achieve the desired biological effects.
27. The use of any one of claims 1 to 26 or the pharmaceutical composition of any one of claims 4 to 26 wherein the applied penetrant dose is between 0.01 mg and 15 mg per nostril, even more often is in the range 0.1 mg and 10 mg per nostril, and preferably is between 0.5 mg and 5 mg per nostril.

28. The use of any one of claims 1 to 27 or the pharmaceutical composition of any one of claims 4 to 27 wherein the efficiency of administration and the biological effects of the agent or drug chosen are controlled by using different application volumes.
29. The use of any one of claims 1 to 28 or the pharmaceutical composition of any one of claims 4 to 28 wherein said formulation is administered using a metered delivery device.
30. The use of any one of claims 1 to 29 or the pharmaceutical composition of any one of claims 4 to 29 wherein different application volumes are selected to control the efficiency of administration and the biological effects of the chosen agent or drug.
31. The use of any one of claims 1 to 30 or the pharmaceutical composition of any one of claims 4 to 30 wherein the penetrants in suspension are loaded with the drugs or agents within 24 hours prior to the formulation administration, preferably 360 min, more preferably 60 min and even more preferably 30 min before the resulting formulation administration in the nose.
32. The use of any one of claims 1 to 31 or the pharmaceutical composition of any one of claims 4 to 31 wherein the delivery device is loaded at the treatment site.
33. The use of any one of claims 1 to 32 or the pharmaceutical composition of any one of claims 4 to 32 wherein the device is loaded separately with penetrants and the molecules, particularly biological agents, to be associated therewith.
34. The use of any one of claims 1 to 33 or the pharmaceutical composition of any one of claims 1 to 33 wherein the pharmaceutically active ingredient is for administration to the nervous system.
35. The use or the pharmaceutical composition of claim 34 wherein the nervous system is the brain.

36. The use of any one of claims 1 to 35 or the pharmaceutical composition according to any one of claims 4 to 35 wherein said pharmaceutical composition is a vaccine.
37. The vaccine of claim 36 which further comprises a pathogen extract or a compound from a pathogen or a fragment or a derivative thereof.
38. The vaccine of claim 37 wherein said pathogen extract or compound is selected from hepatitis virus, (human) immunodeficiency virus, herpes viruses, small-pox (chicken-pox), influenza, measles, mumps or polio viruses, cytomegalovirus, rhinovirus, etc., or fungi prospering inside host cells, a parasite including animal parasites, such as protozoa and helminths, and ectoparasites, such as ticks and mites, or *Brucella* species, including the causative agent for cholera, Haemophilus species, as well as pathogens triggering paratyphoid, plague, rabies, tetanus or rubella diseases.
39. The vaccine of any one of claims 36 to 38 which further comprises an adjuvant.
40. The vaccine of claim 38 or 39 wherein said adjuvant is lipopolysaccharide, such as lipid A or a derivative or modification thereof, such as monophosphoryl lipid A, or its analogue, such as a fatty derivative of saccharose, cord-factor (trehalose-dimycolate), muramyl dipeptide, or another (poly)saccharide or (poly)peptide identical to or resembling an immunologically active part of a membrane of a microorganism; an extract of a microorganism, including bacterial exo- and endotoxins, preferably cholera toxin or the heat labile toxin of *E. coli*, an A-chain derivative, a component with an ADP-ribosylating activity, a peptidoglycane, a clostridial toxin, an LT halotoxin, purified protein derivative of *M. tuberculosis*, LT-R192G, Fibronectin-binding protein I of *Streptococcus pyrogenes*, or outer membrane protein of group B *Neisseria meningitidis* (GBOMP), bacterial or viral nucleic acids, such as oligonucleotides comprising unmethylated CpG dinucleotides.
41. The vaccine of any one of claims 36 to 40 comprising a blend of MPL and IL-12 or GM-CSF and IL-4.

42. The vaccine of any one of claims 36 to 41 wherein the relative immunogen/antigen dose to be administered non-invasively through the nose by means of highly adaptable carriers is chosen to be between 0.01x and 100x, more often between 0.05x and 75x, and even more preferably between 0.1x and 50x different from the corresponding immunogen/antigen dose that would have to be injected to achieve the desired biological effect.
43. The vaccine according to any one of claims 39 to 42 wherein the concentration of the transnasally administered adjuvant is between 10x lower and up to 1000x higher than that used with the corresponding subcutaneously injected formulations employing similar antigen, the transnasally administered immunoadjuvant concentration more often differing from the injected immunoadjuvant concentration by the factor between 0.5 and 100, or better, by the factor between 1 and 50, and best between 2 and 25.
44. A container comprising the pharmaceutical composition according to any one of claims 4 to 43.
45. A package comprising at least one container comprising the pharmaceutical composition of any one of claims 4 to 43.
46. A method for generating a protective immuno response on a mammal by vaccinating said mammal with a vaccine according to any one of claims 36 to 43.
47. The method according to claim 46 wherein different administration volumes are selected to control the applied immunogen dose and the outcome of vaccination.
48. The method according to claim 46 or 47, wherein a suspension of antigen-free penetrants is loaded with the antigen to be associated therewith during the day prior to an administration, preferably 360 min, more preferably 60 min and even more preferably 30 min before administering the resulting formulation in the nose.

49. The method of any one of claims 46 to 48 characterised in that at least one dose of vaccine is administered.
50. The method according to claim 49 wherein said vaccine is administered as a booster vaccination.
51. The method according to any one of claims 46 to 50, wherein the vaccine is applied between 2 and 10, preferably between 2 and 7, even more preferably up to 5 and most preferably up to 3 times, when a non-allergenic antigen is used, or such a number of times, in the case of allergens, as is required either to achieve the desired immuno-tolerance, determined according to a suitable assessment method, or else to deem the effort as having failed.
52. The method according to any one of claims 48 to 51, wherein the time interval between the subsequent vaccinations is chosen to be between 2 weeks and 5 years, often between 1 month and up to 3 years, more frequently between 2 months and 1.5 years.
53. The method according to any one of claims 46 to 52, wherein the flux of penetrants that carry an immunogen through the various pores in a well-defined barrier is determined as a function of a suitable driving force or a pressure acting across the barrier and the data are then conveniently described by a characteristic curve which, in turn, is employed to optimise the formulation or application further.



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Intranasal application of Transfersulin  
IDDM test person, -0.8 IU/kg

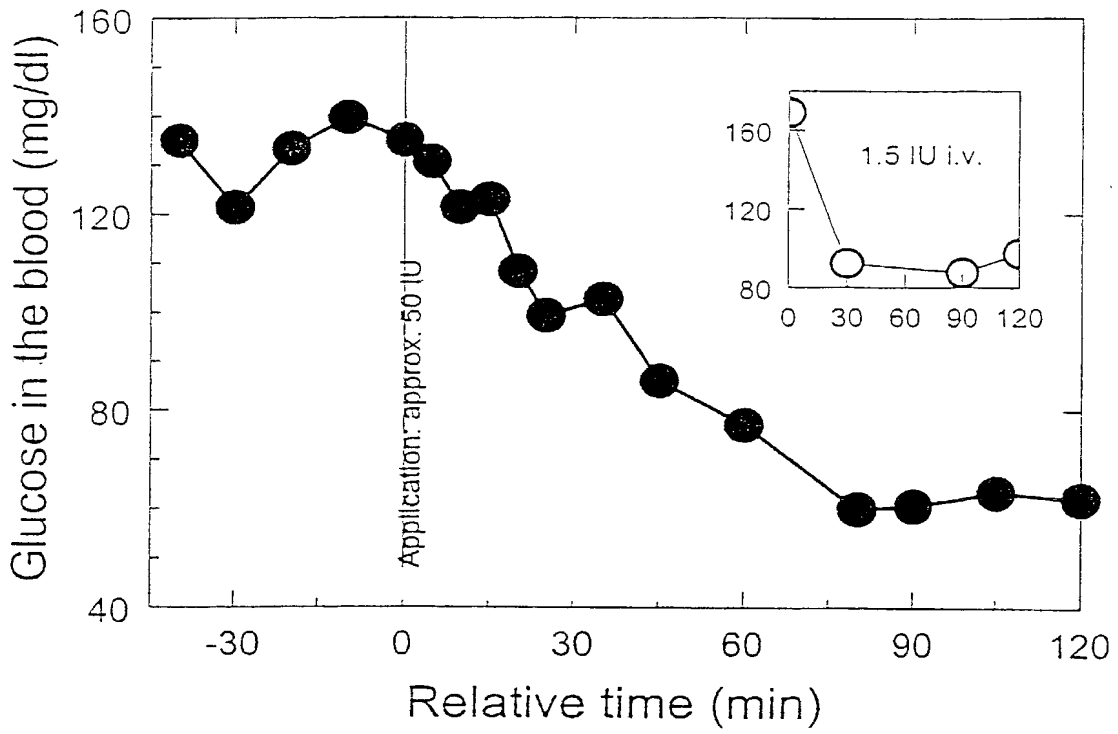


Fig. 1



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Intranasal application of Transfersulin  
 on a normoglycaemic test person, ~ 0.15 IU/kg (2x)

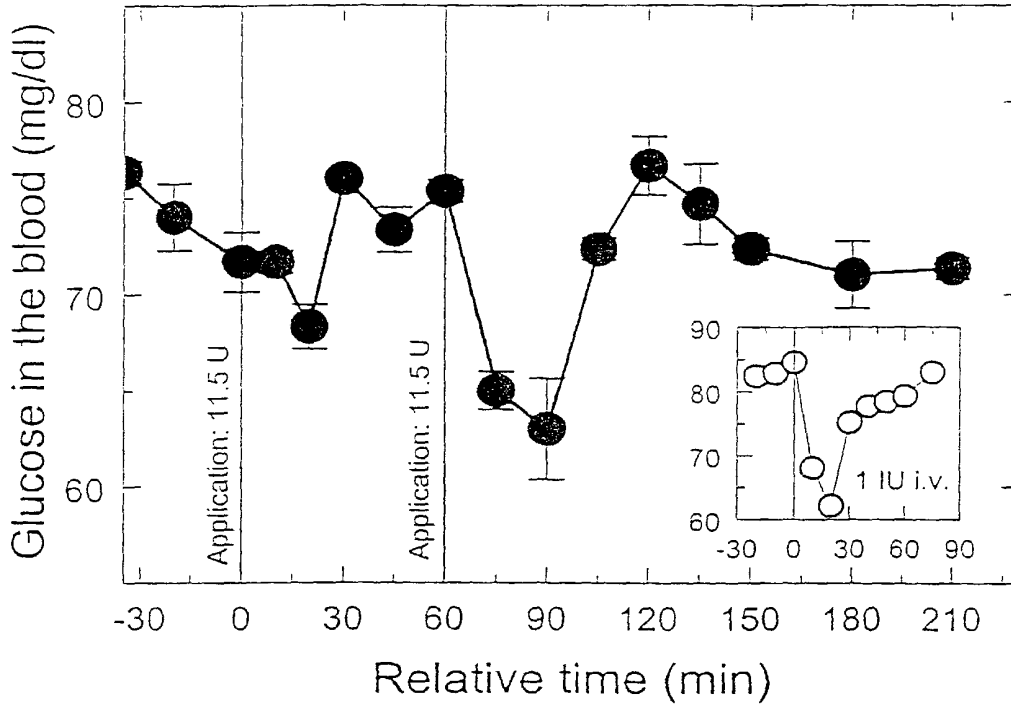


Fig. 2

3/24

Intranasal application of Transfersulin  
on a normoglycaemic test person, ~ 0.15 IU/kg (2x)

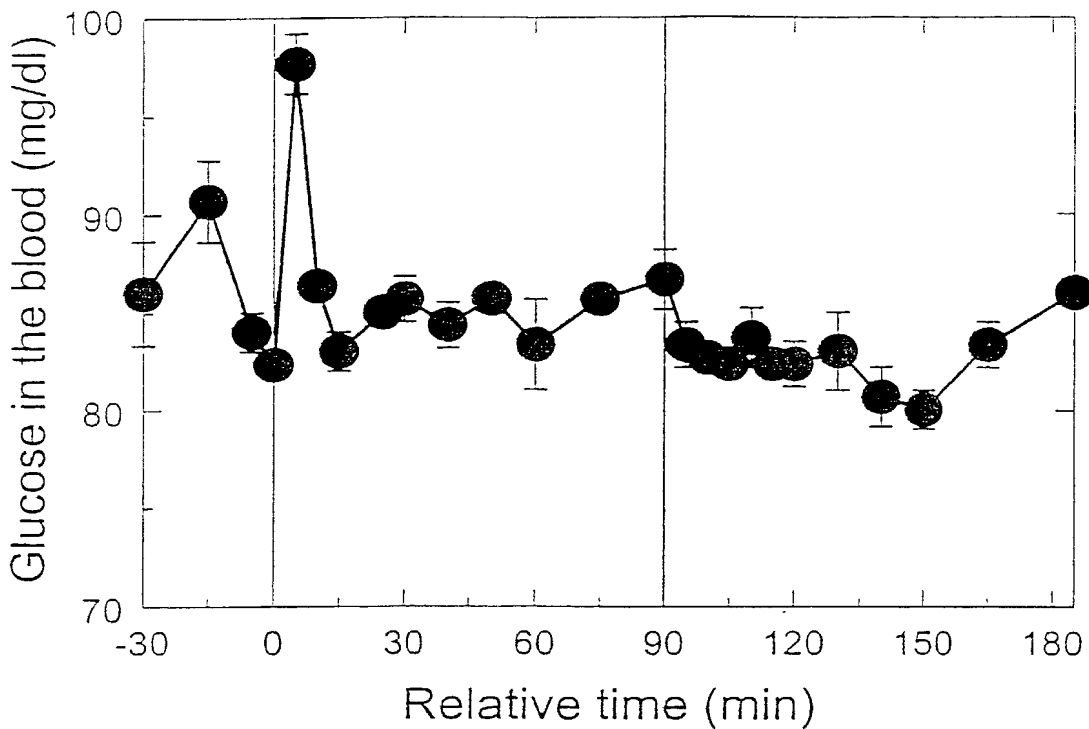


Fig. 3a

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Intranasal application of Transfersulin  
in IDDM test person, -0.4 IU/kg

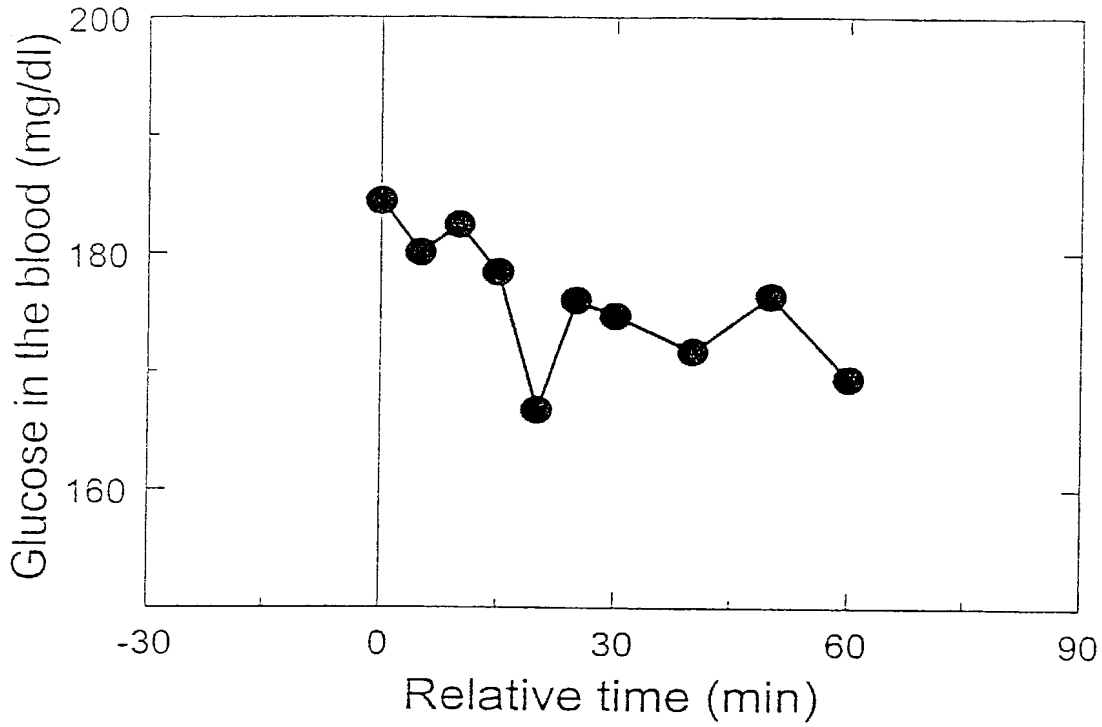


Fig. 3b

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Effects of nasally administered  
cytokines on specific immune response,  
1st boost + 7 d

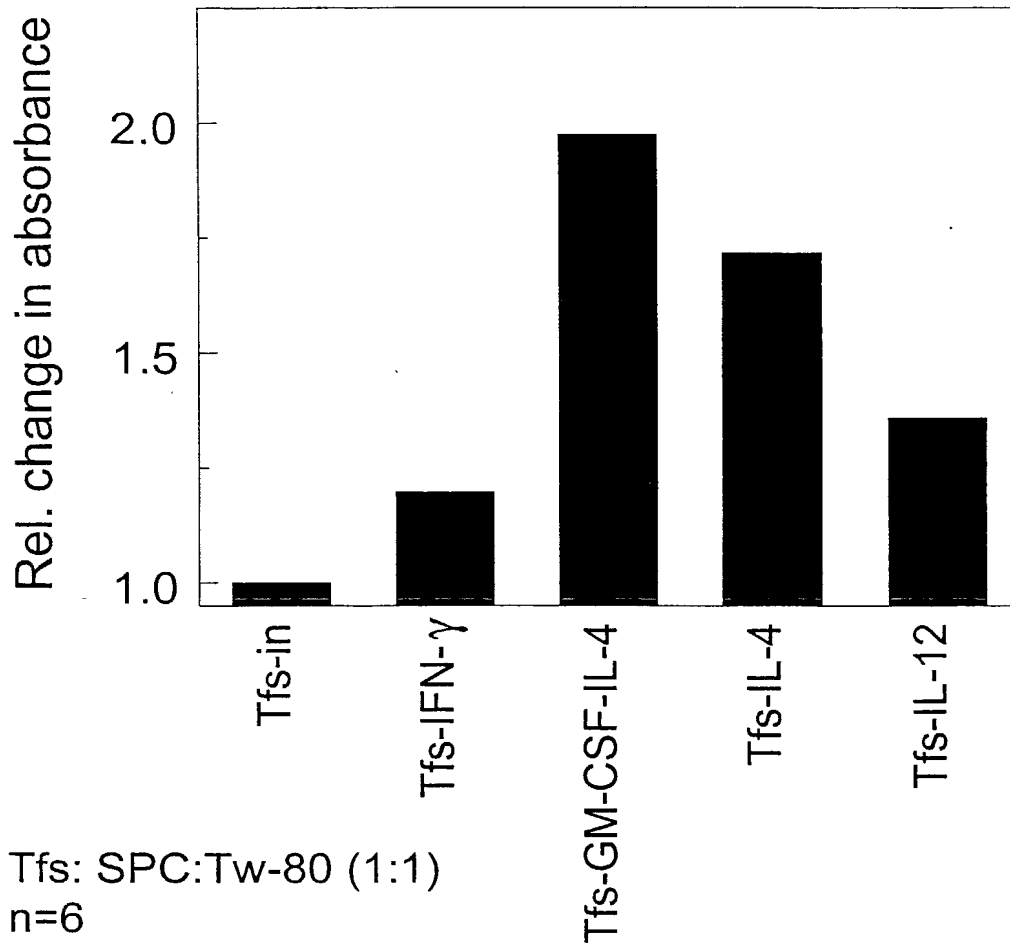


Fig. 4

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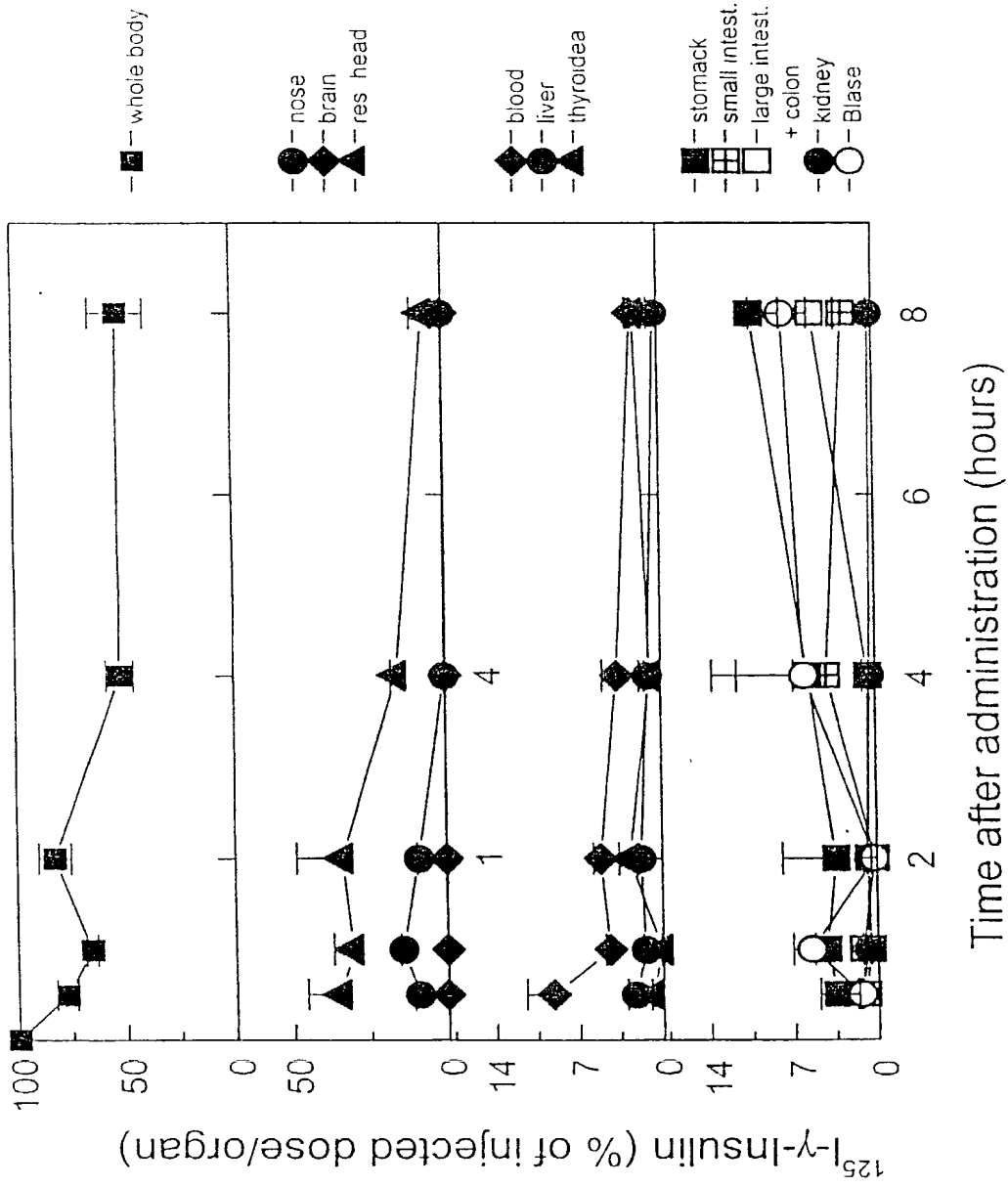


Fig. 5

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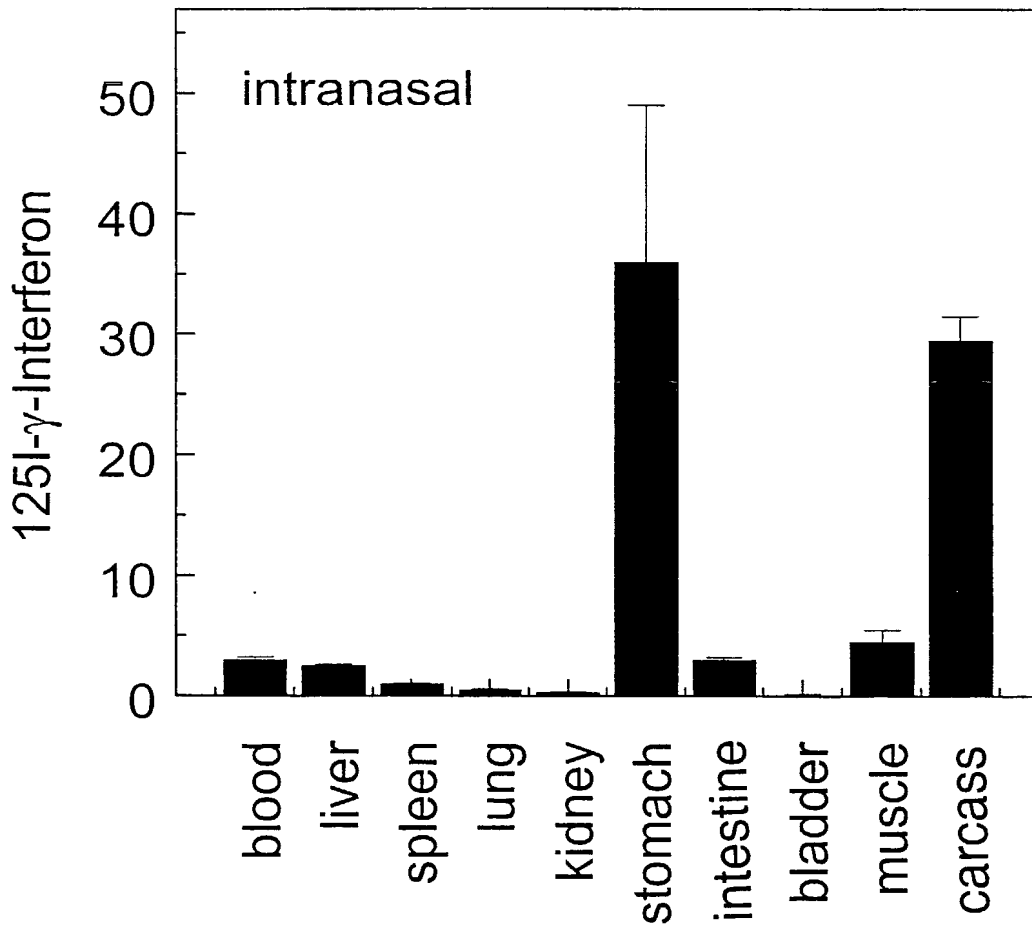


Fig. 6

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Intranasal administration: Tetanus toxoid (TT, impure)

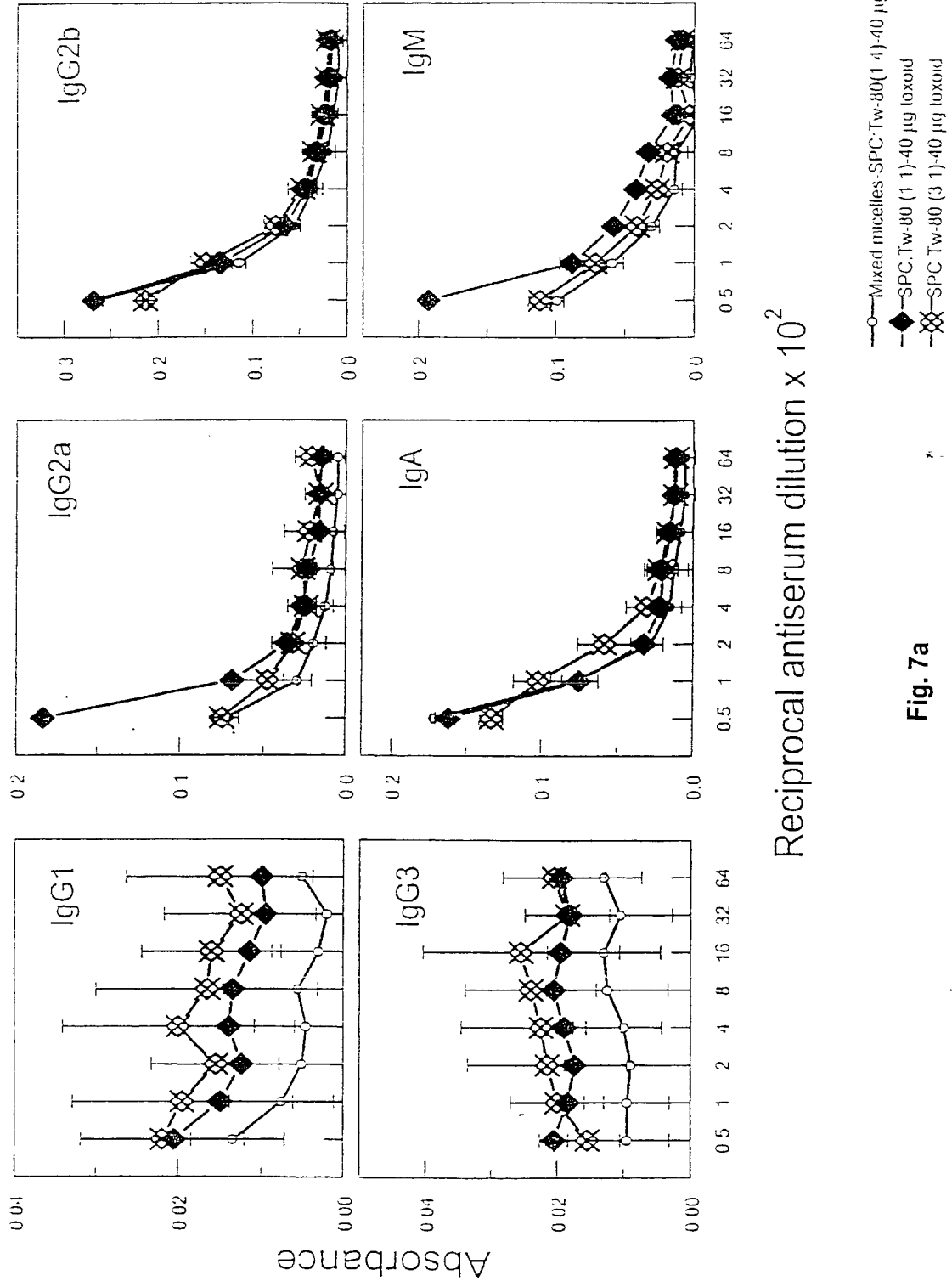
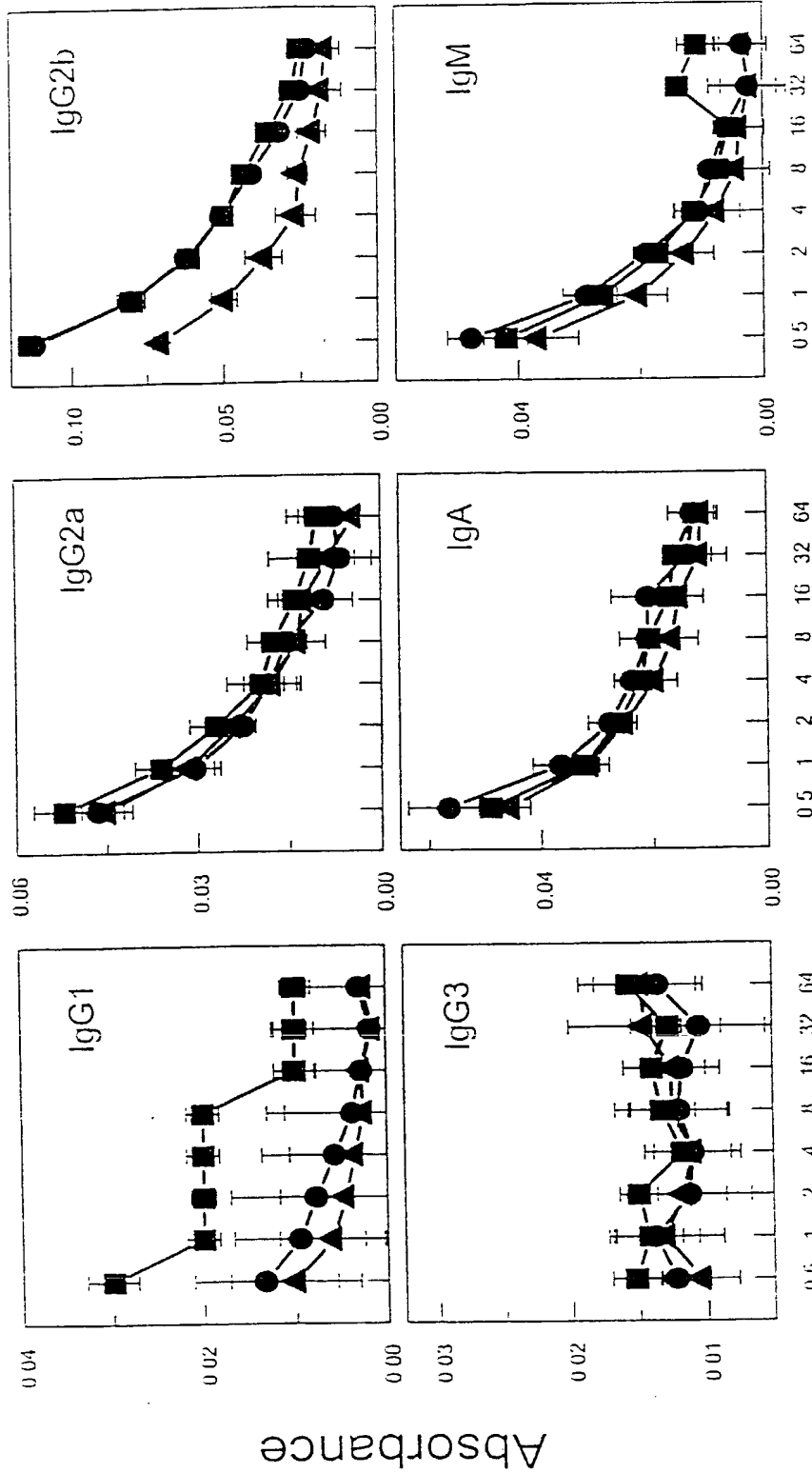


Fig. 7a

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Intranasal immunisation: Tetanus toxoid (TT impure)



Reciprocal antiserum dilution x 10<sup>2</sup>

- SPC:NaCh (4.5 l)
- Mixed micelles (SNh 1.1)
- ▲ SPC Cholesterol (1 1)

Antigen dose in all the groups : 40 µg per mouse

Fig. 7b



**Intranasal immunisation: Tetanus toxoid (TT, impure)**  
**Effect of carrier size and deformability**

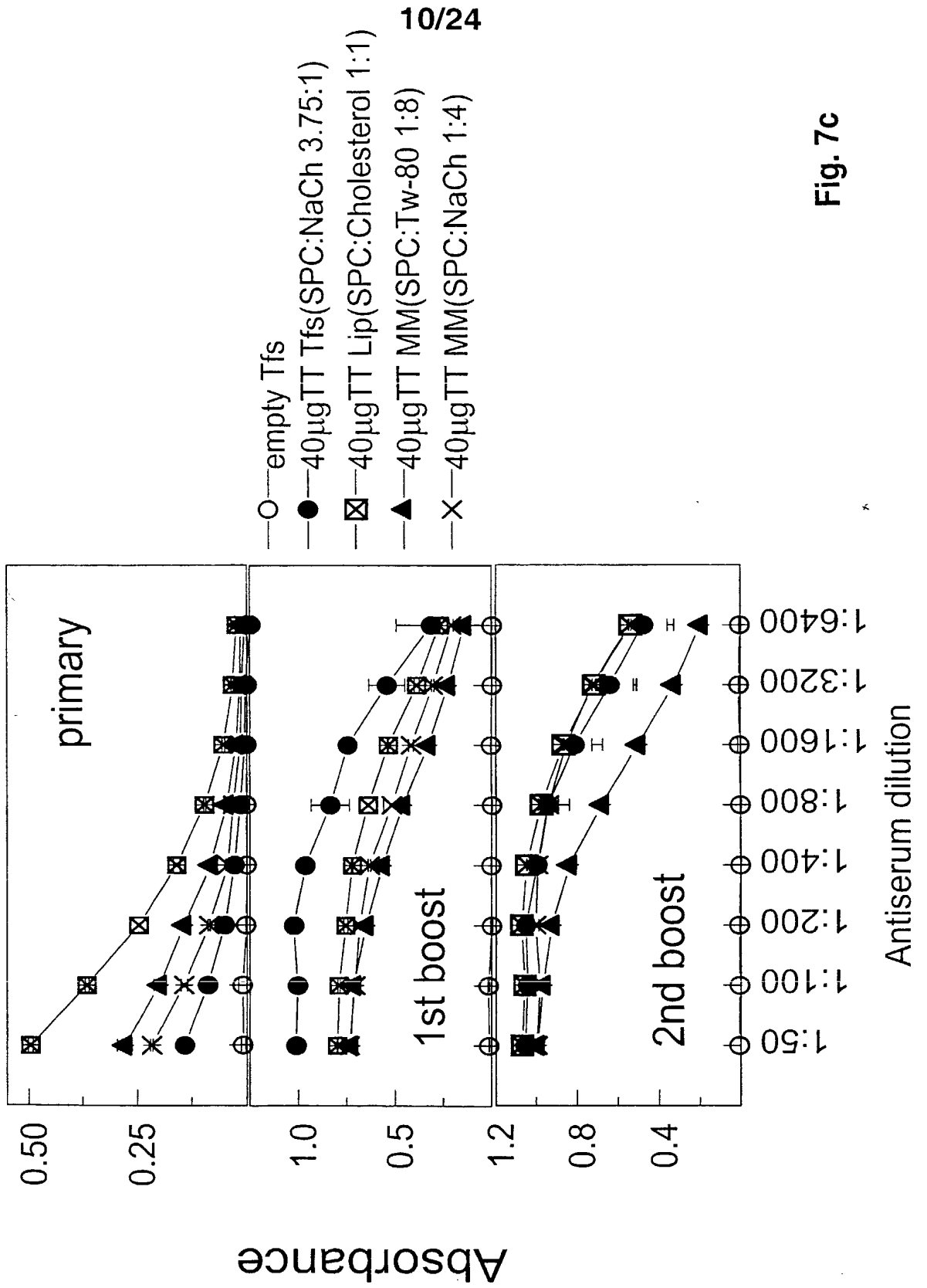


Fig. 7c

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Intranasal Immunization: Tetanus toxoid, (TT, purified)  
dose effect

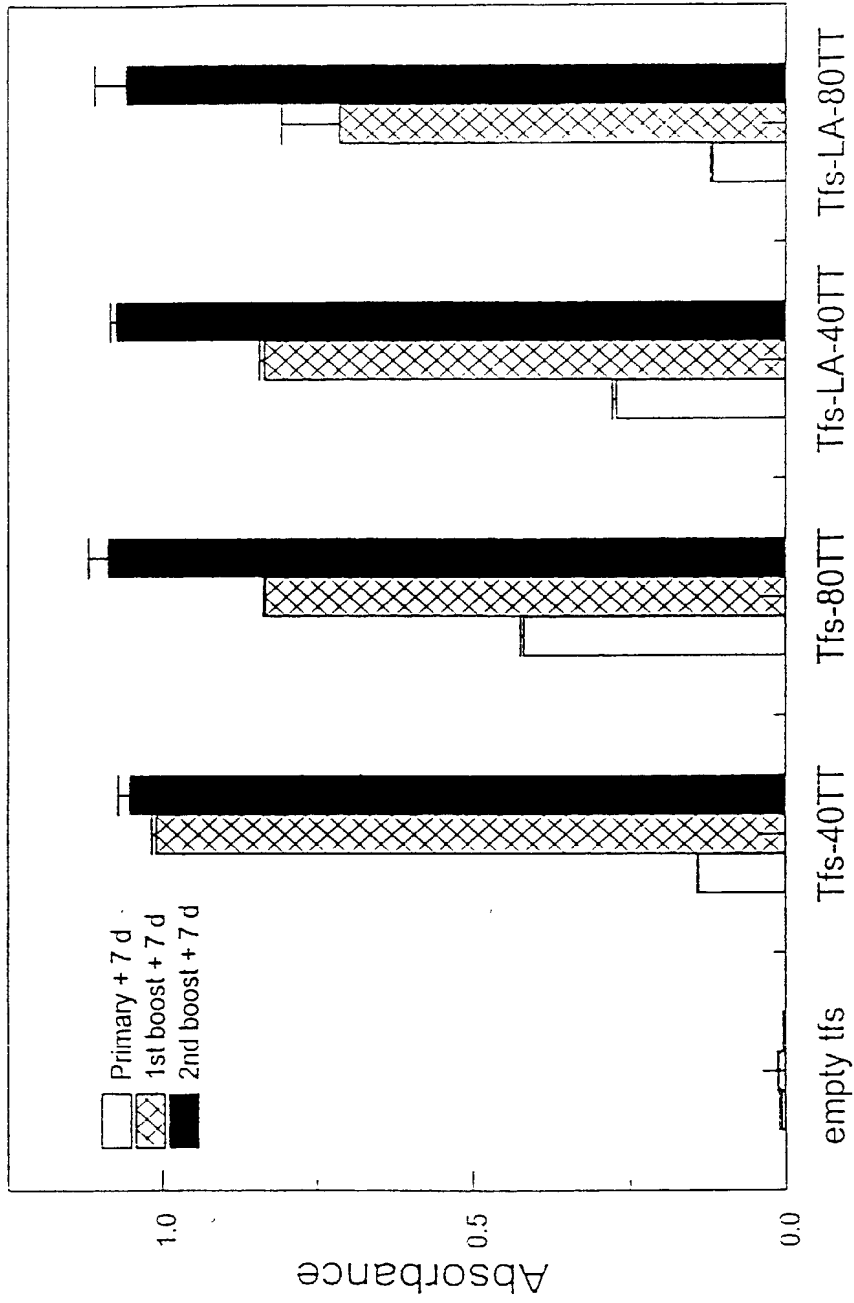


Fig. 8a

Intranasal immunisation: Tetanus toxoid (TT, purified), Effect of antigen dose

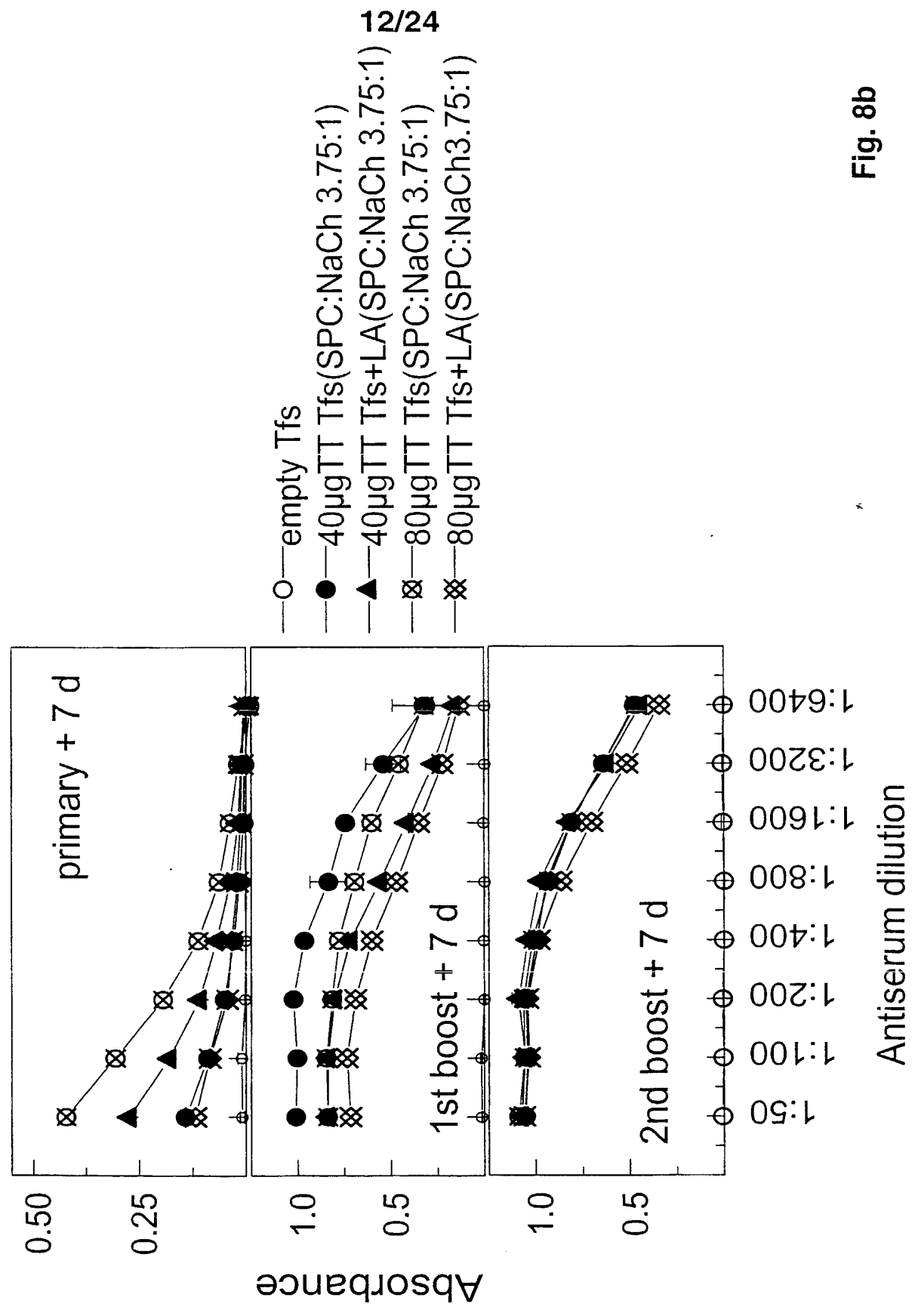


Fig. 8b

Intranasal Immunization: Tetanus toxoid (TT, purified), dose effect

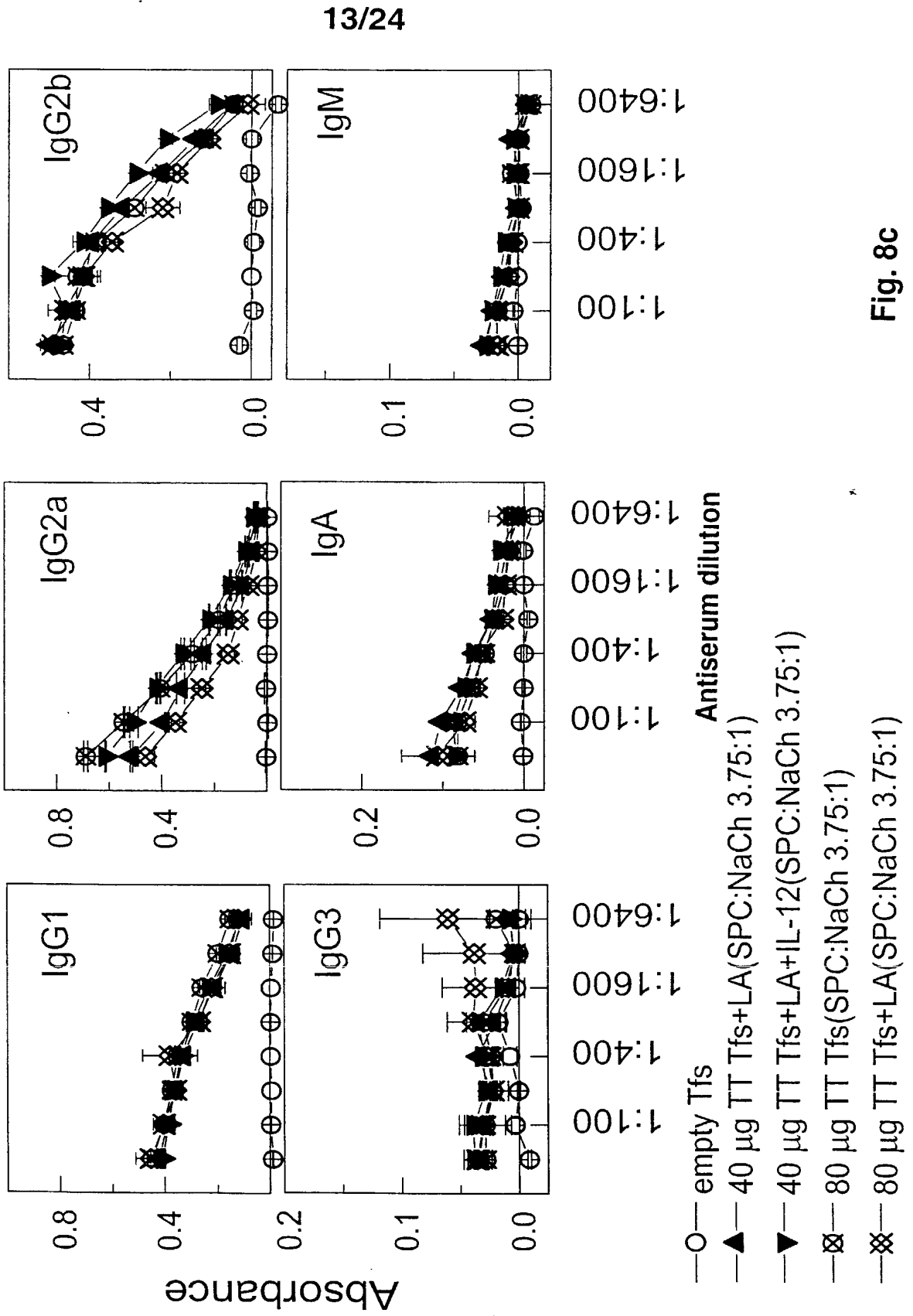


Fig. 8c

Noninvasive immunisation: Tetanus toxoid (TT, purified)  
effect of application route

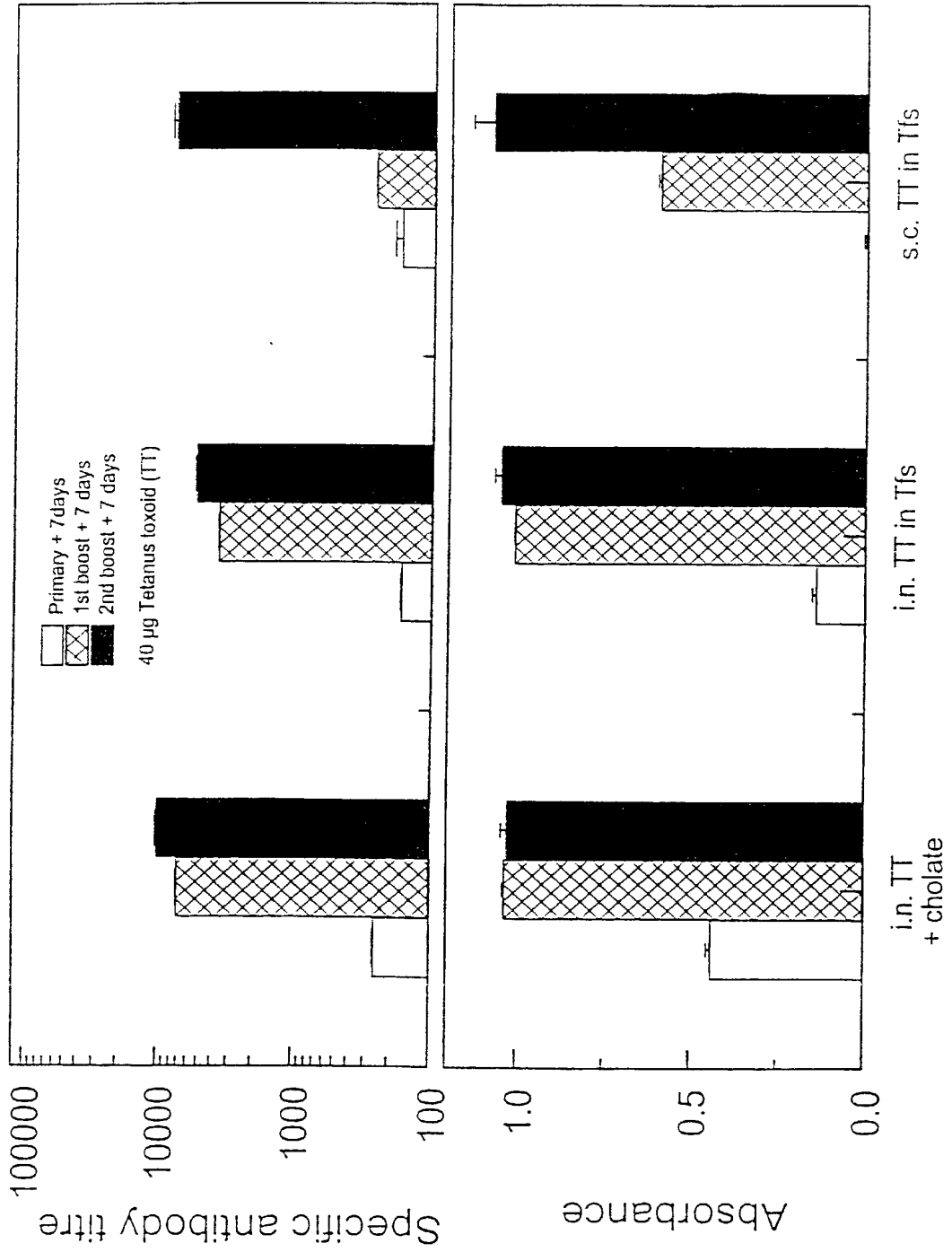


Fig. 9a

# Noninvasive immunisation: Tetanus toxoid (TT, purified) route of application effect

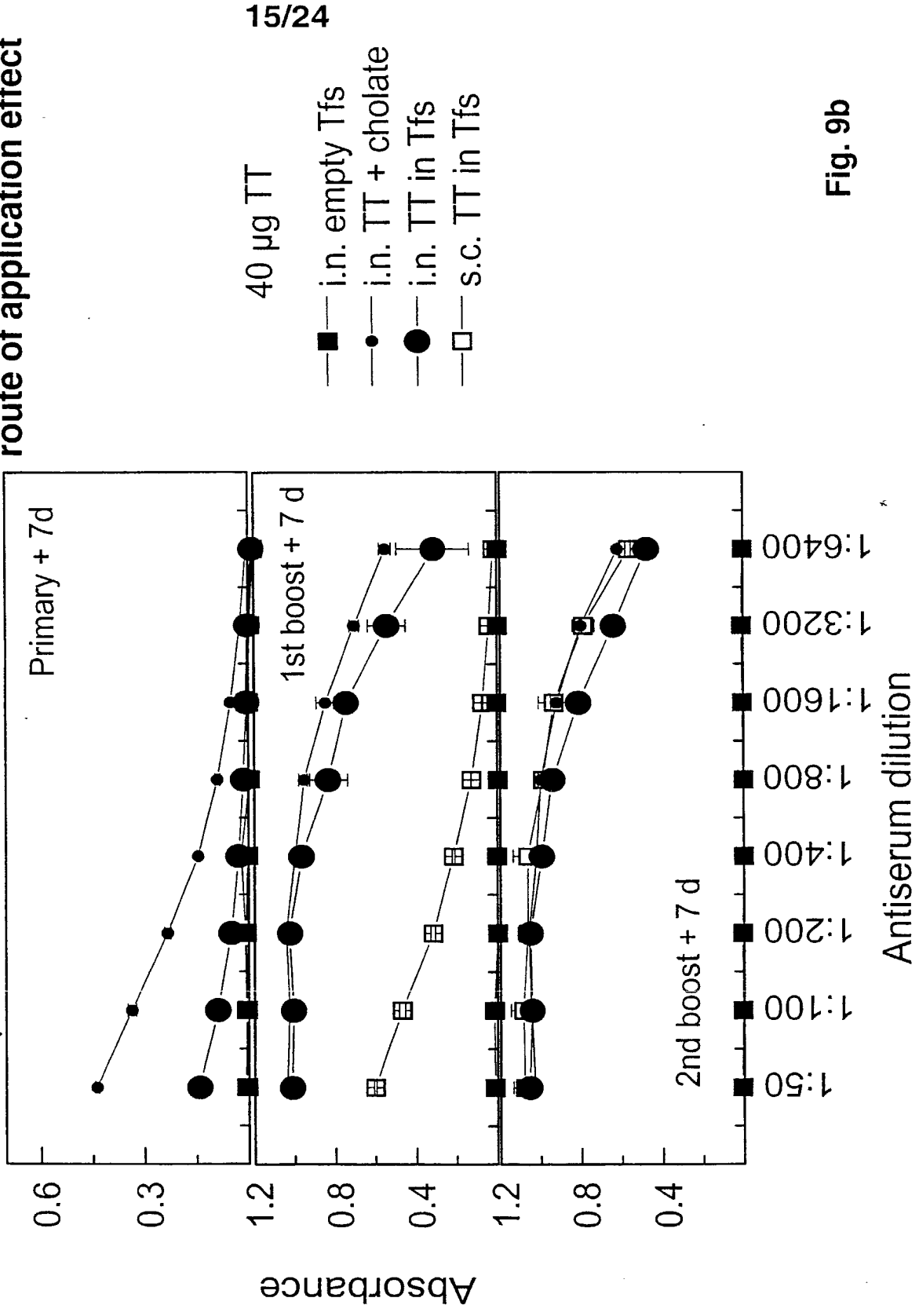


Fig. 9b

Antigen-specific Isotyping: Intranasal / Subcutaneous

40 µg Tetanus Toxoid (TT), ultrafiltered

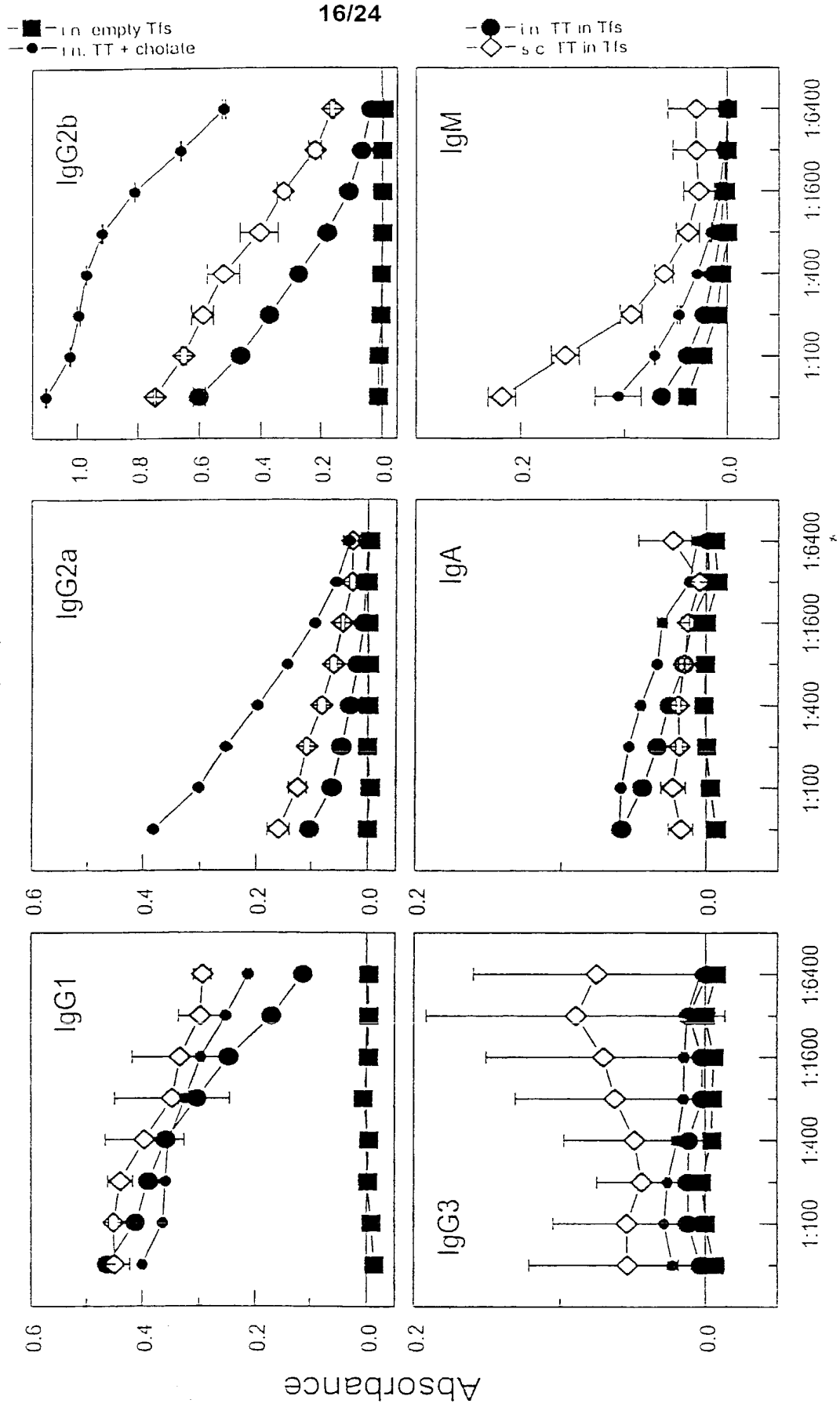


Fig. 9c

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### Intranasal immunisation: Tetanus toxoid (TT)

dose and route of administration effect

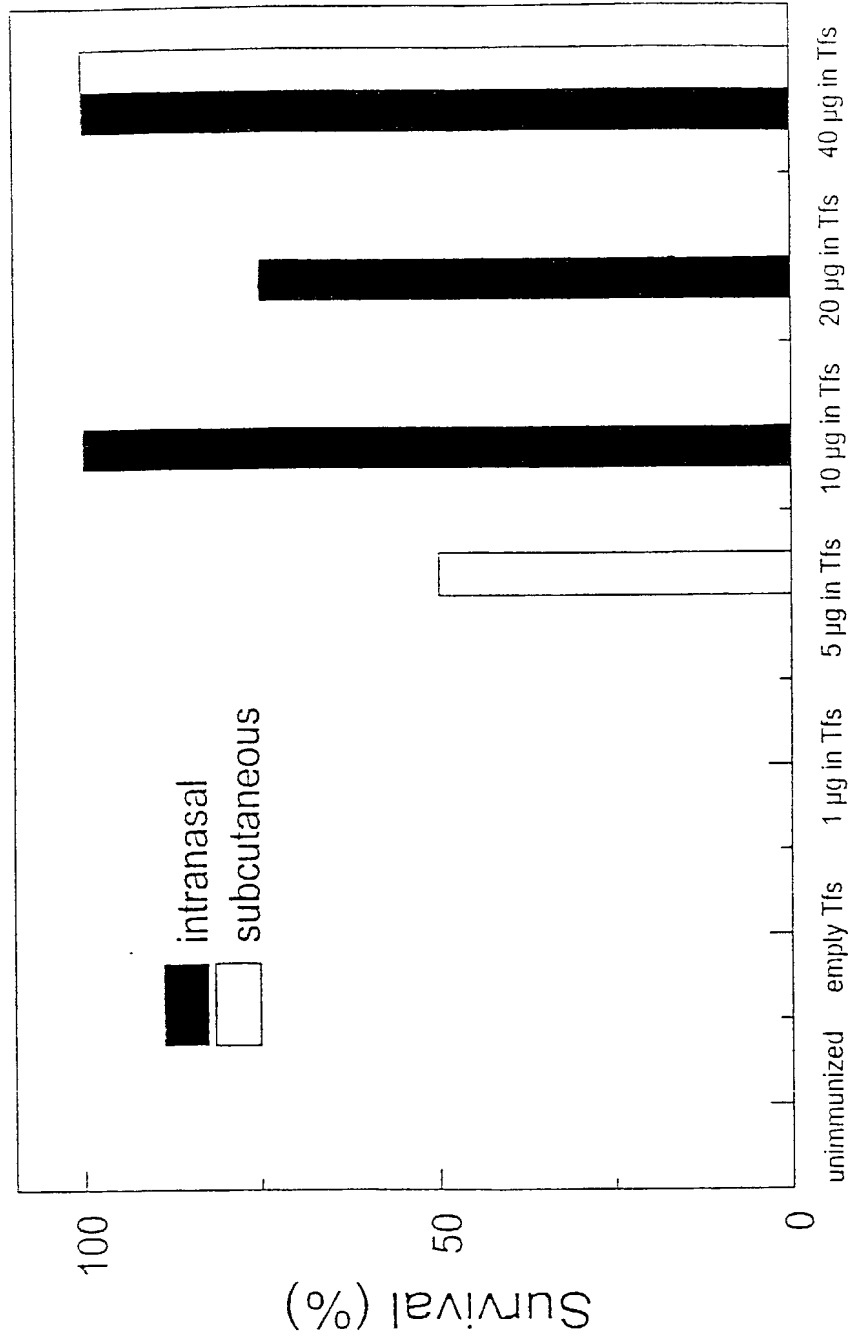


Fig. 10a



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Intranasal immunisation: Tetanus toxoid (TT, purified) dose effect

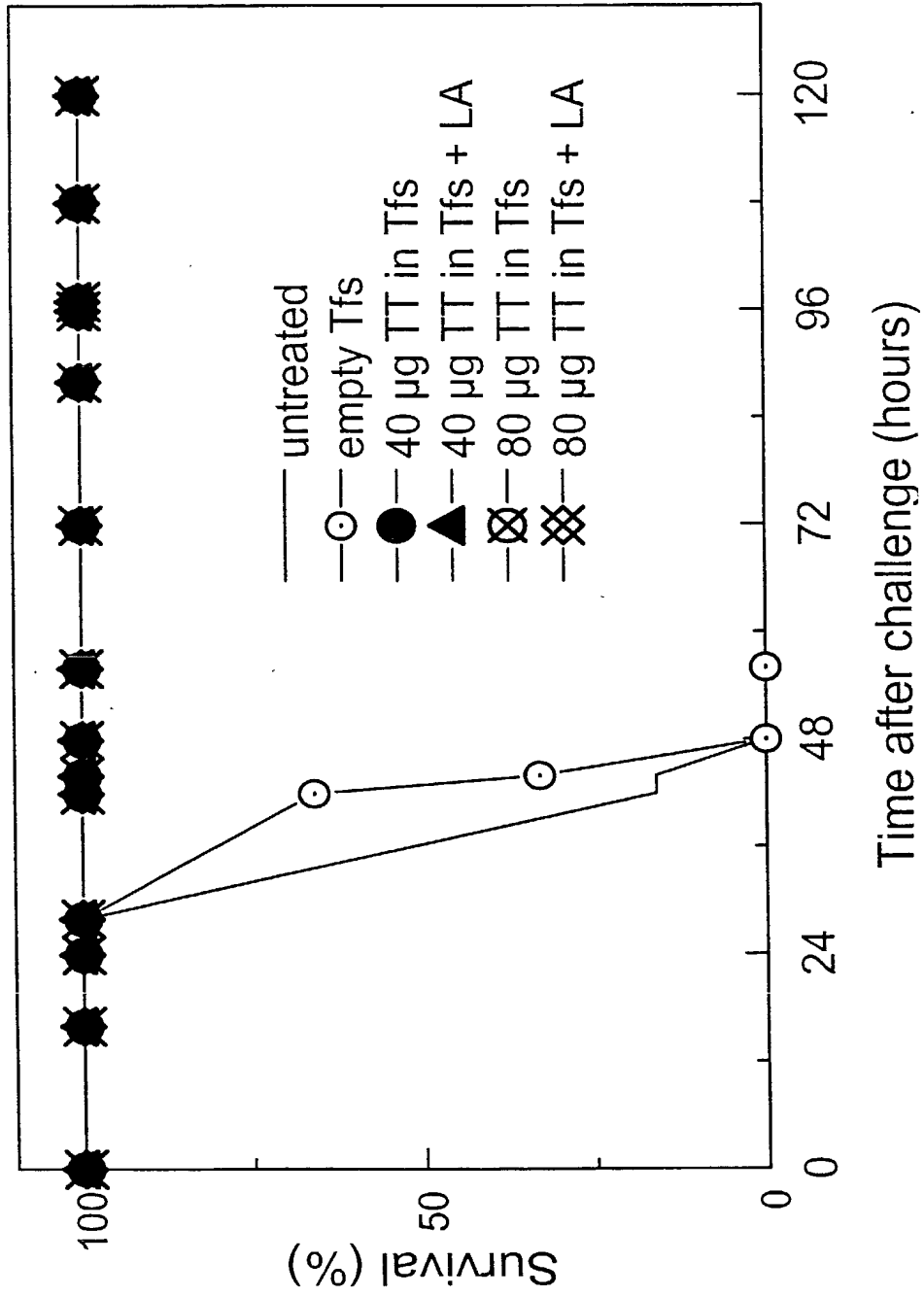


Fig. 10b

### Intranasal immunisation: Tetanus toxoid (TT, impure) effect of immunomodulators

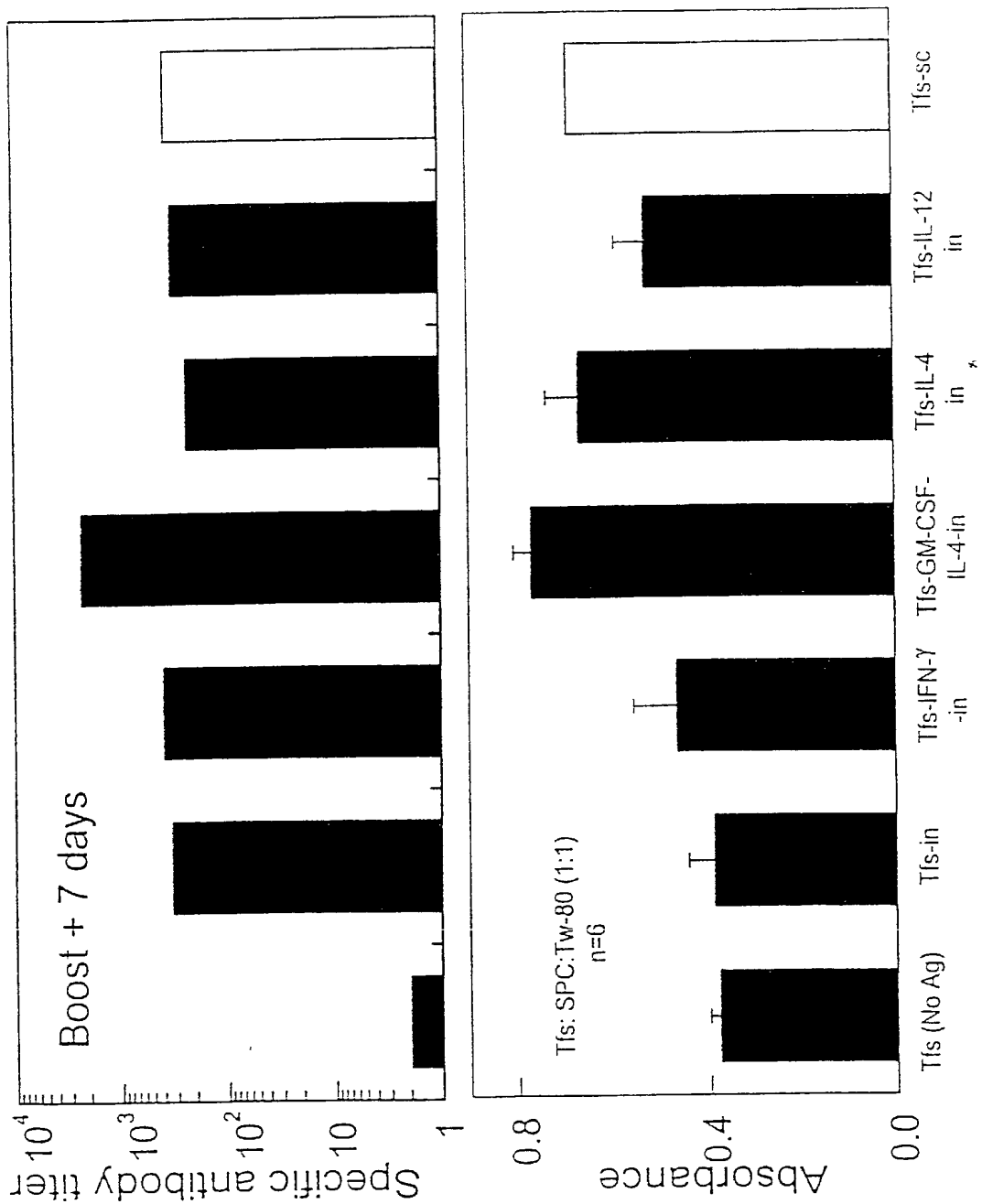
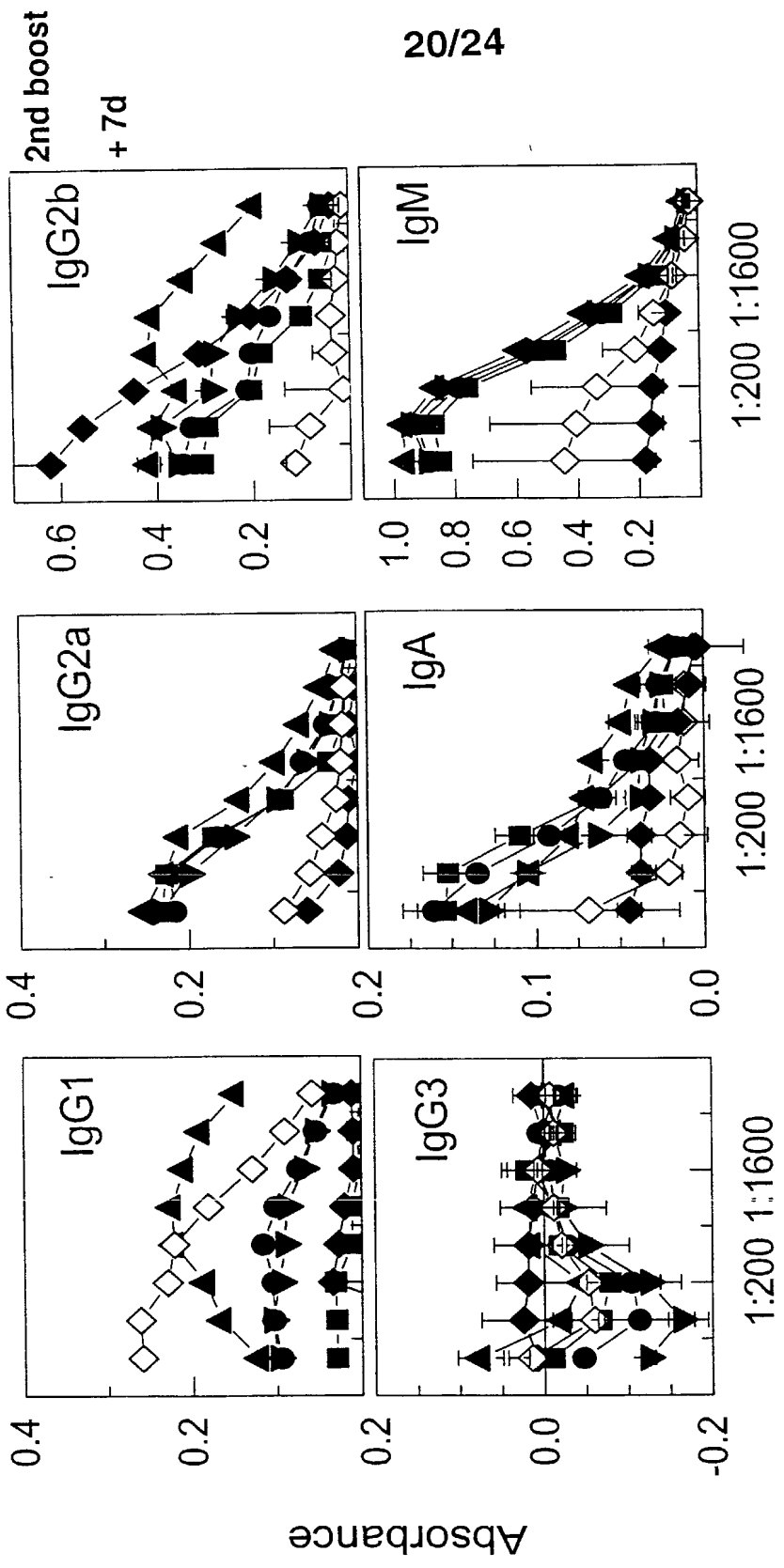


Fig. 11a

Intranasal immunisation: Tetanus toxoid (TT, impure, 40 µg), effect of immunomodulators, 20/24



- ◇ i.n. TT in Tfs
  - i.n. TT in Tfs + IFN-γ
  - ▲ i.n. TT in Tfs + GM-CSF + IL-4
  - ▼ i.n. TT in Tfs + IL-4
  - ◆ i.n. TT in Tfs + IL-12
  - s.c. TT in Tfs
- Antiserum dilution
- SPC:Tw-80 3.5:1

Fig. 11b

Intranasal immunisation: Tetanus toxoid (TT, purified)  
effect of immunopotentiators

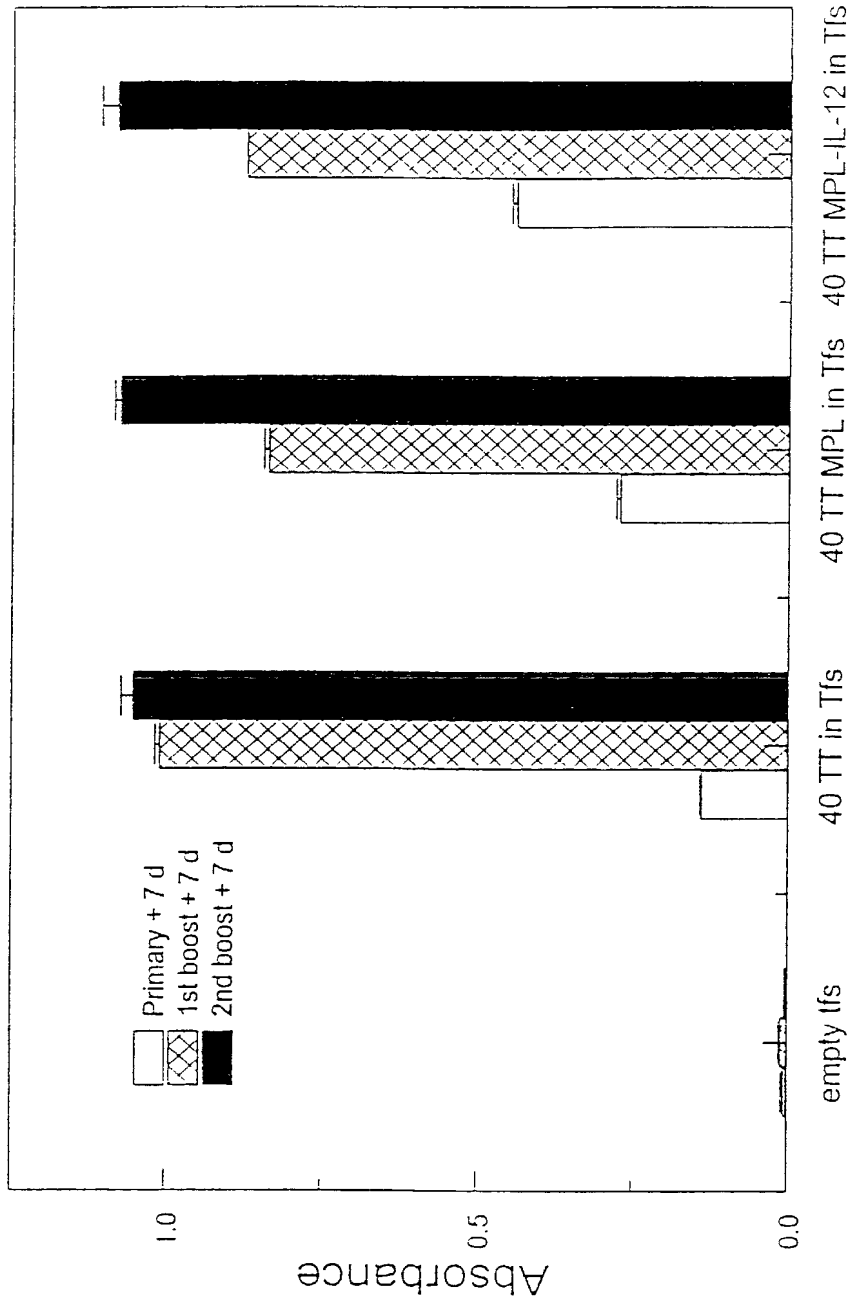
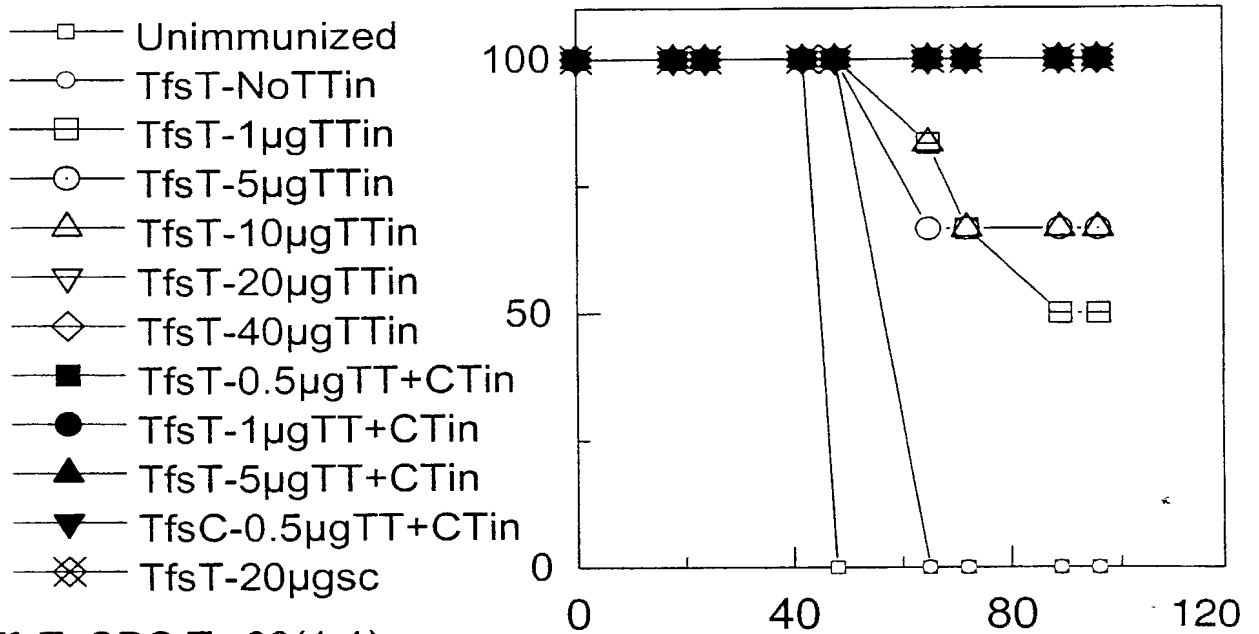


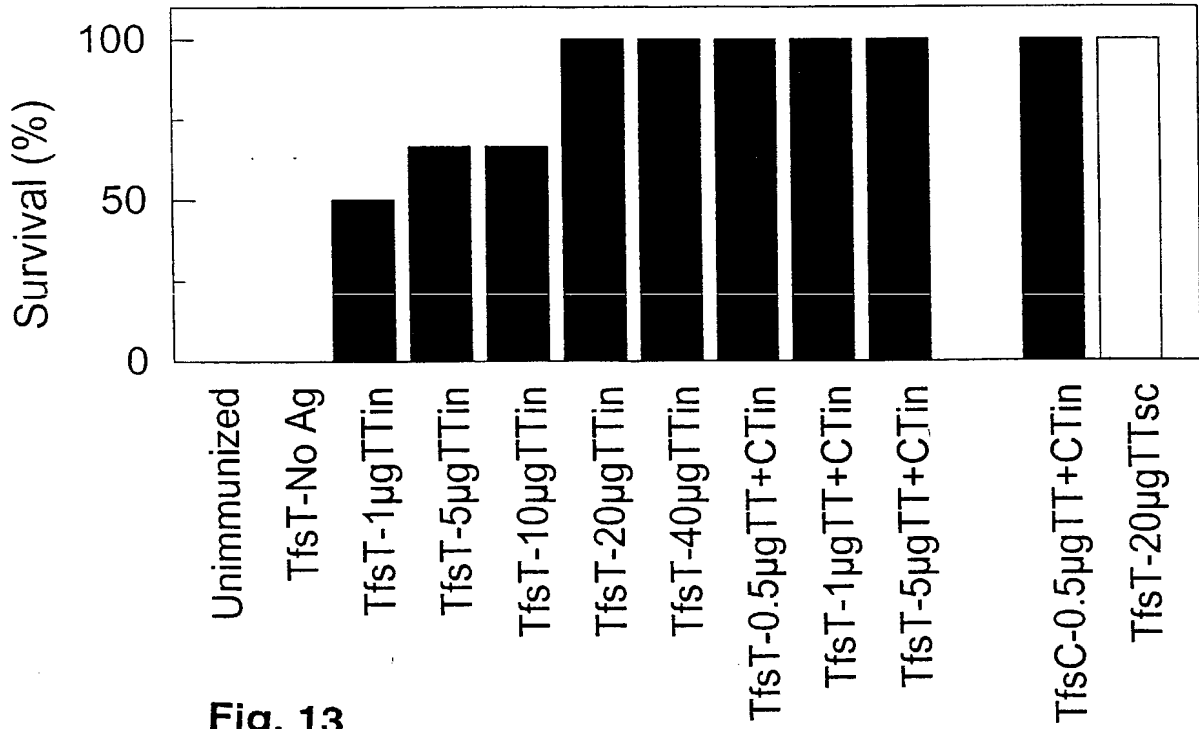
Fig. 12

\*

**22/24**  
**Potential of nasal immune response to tetanus**  
**toxoid (TT) by bacterial adjuvant, cholera toxin (CT)**



TfsT: SPC:Tw80(1:1)  
 TfsC: SPC:NaCholate(3.75:1)  
 CT dose: 2µg per dose



**Fig. 13**

# Adjuvant effect: of Heat Labile Toxin (LT) from E.coli

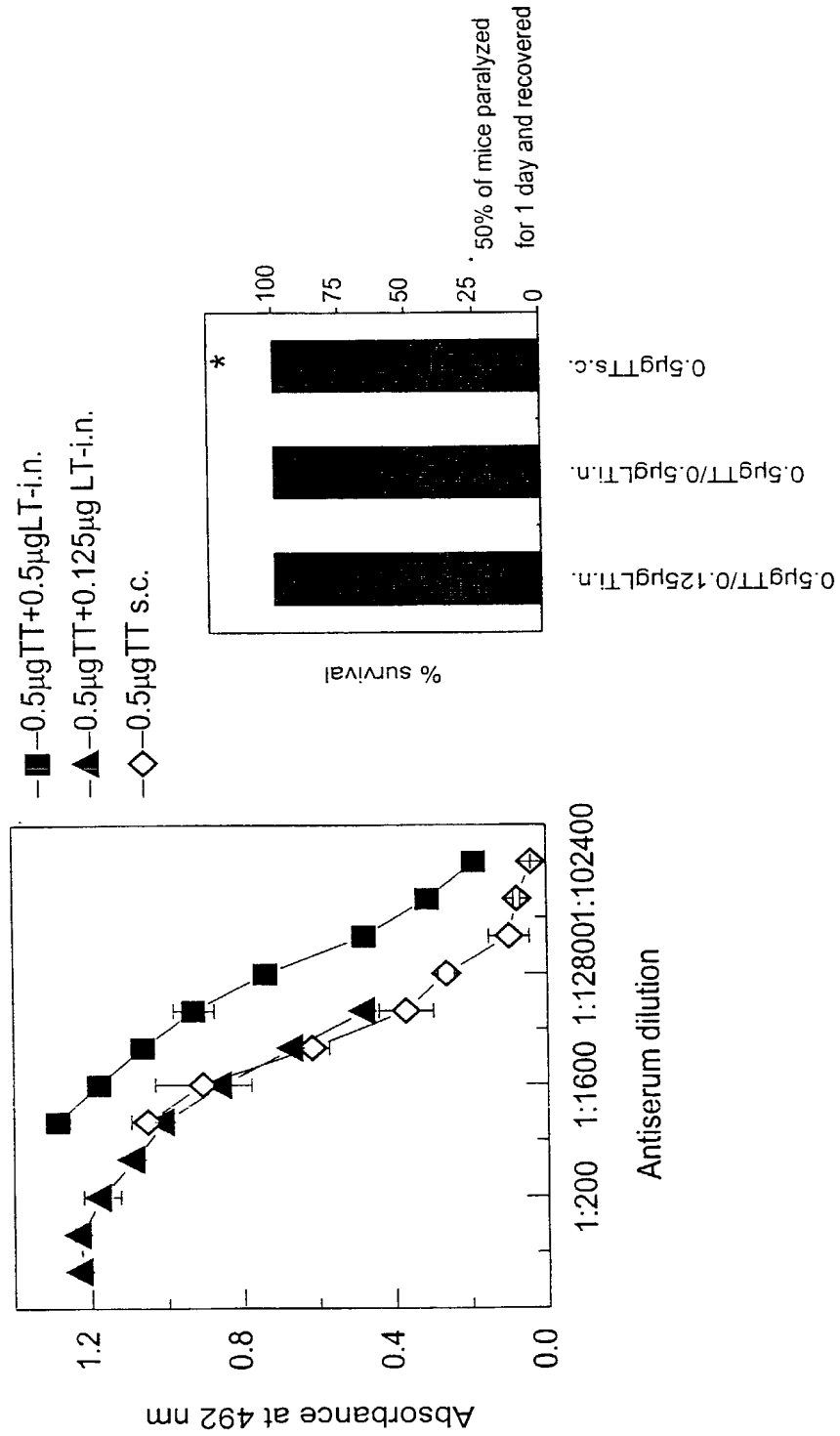


Fig. 14

**Bi-Valent Vaccines: Anti-Tetanus and -Cholera response**  
to the antigens administration in Transfersomes in the nose

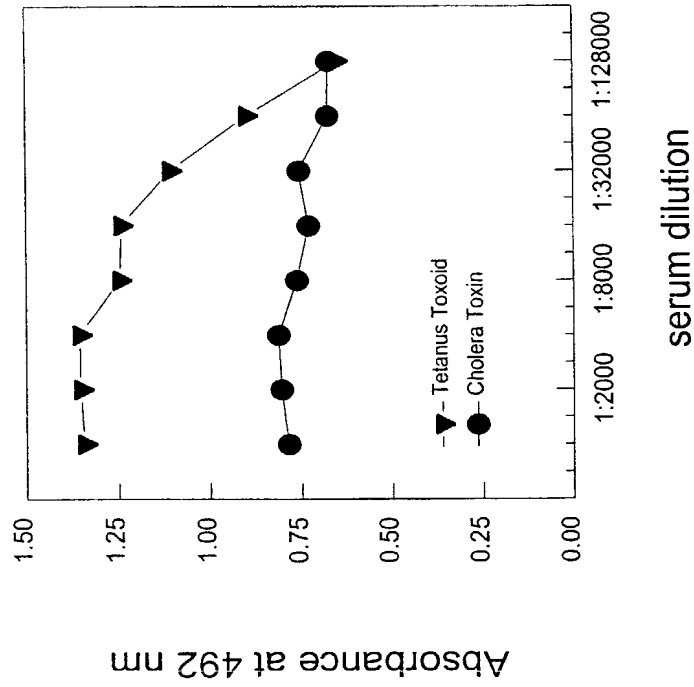


Fig. 15



13 Rec'd PCT/PTO U.S. APR 2002  
09/890371

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor(s): **GREGOR CEVC, et al**      Group Art Unit: **TBA**  
Appln. No. 09/890,371      Examiner: **TBA**  
Filing Date: **JULY 26, 2001**      Atty Dkt **009848/0272496**  
Title: **TRANSNASAL**      Date: March 28, 2002  
**TRANSPORT/**  
**IMMUNISATION WITH**  
**HIGHLY ADAPTABLE**  
**CARRIERS**

\* \* \* \* \*

**CHANGE OF ADDRESS NOTICE**  
**for Filed Application**

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Washington, D.C. 20231

Sir:

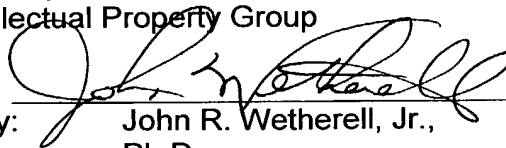
Effective immediately, please change the correspondence address to:

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Respectfully submitted,

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PAT-210 6/01





Docket No. 009848/027 2496

# Declaration and Power of Attorney For Patent Application

## English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled  
**TRANSNASAL TRANSPORT/IMMUNISATION WITH HIGHLY ADAPTABLE CARRIERS**

the specification of which

(check one)

- is attached hereto.
- was filed on July 26, 2001 as United States Application No. or PCT International Application Number 09/890,371 and was amended on \_\_\_\_\_ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Not Claimed
<u>99101480.4</u> (Number)	<u>EP</u> (Country)	<u>01/27/99</u> (Day/Month/Year Filed)	<input type="checkbox"/>
_____ (Number)	_____ (Country)	_____ (Day/Month/Year Filed)	<input type="checkbox"/>
_____ (Number)	_____ (Country)	_____ (Day/Month/Year Filed)	<input type="checkbox"/>

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

_____	_____
(Application Serial No.)	(Filing Date)
_____	_____
(Application Serial No.)	(Filing Date)
_____	_____
(Application Serial No.)	(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

_____	_____	_____
PCT/EP00/00598	26 January 2000	(Status)
(Application Serial No.)	(Filing Date)	(patented, pending, abandoned)
_____	_____	_____
(Application Serial No.)	(Filing Date)	(Status)
(Application Serial No.)	(Filing Date)	(patented, pending, abandoned)
_____	_____	_____
(Application Serial No.)	(Filing Date)	(Status)
(Application Serial No.)	(Filing Date)	(patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

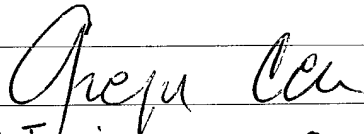
POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (list name and registration number)

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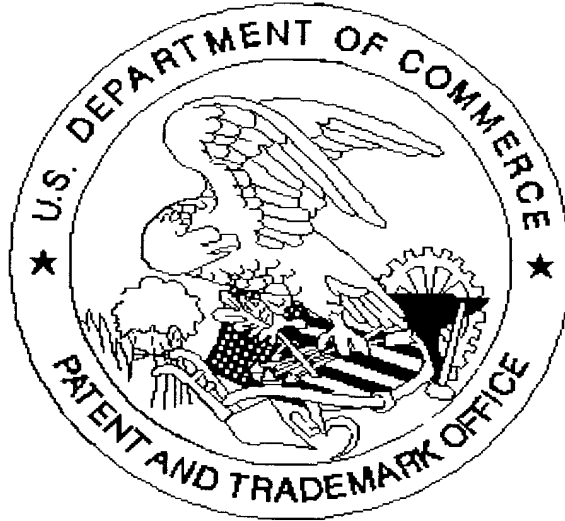
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